THE ROLE OF TREFOIL FACTOR 3 IN MAMMARY CARCINOMA ANGIOGENESIS

LAU WAI HOE

M.Sc. (Pharmacy), USM, Malaysia

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
DEPARTMENT OF PHARMACOLOGY
NATIONAL UNIVERSITY OF SINGAPORE

2014

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously

_____

Lau Wai Hoe
22 August 2014

Acknowledgment

My heartfelt thanks to God for His love and grace that enabling me to accomplish my PhD degree and walked through this journey.

First and foremost, I am gratitude to my supervisor Professor Peter E. Lobie for providing guidance and intellectual support throughout this research project. I also would like to express my thanks to Associate Prof. Zhu Tao and his research team for the collaboration works in xenograft studies.

I am thankful to Thesis Advisory Committee (TAC), Dr. Alan Prem Kumar, Dr. Gautam Sethi and Prof. Peter E. Lobie for their constructive suggestions and inputs in the TAC meetings. Additionally, my gratitude extended to Dr. Lim Yoon Pin, Dr. Gautam Sethi, Prof. Takashi Tokino, and Dr. Suswam A. Esther for generously providing endothelial cell line and plasmids for my research project.

I am grateful to Yong Loo Lin School of Medicine (SoM), National University of Singapore for offering me the Graduate Research Scholarship to pursue my PhD degree. Furthermore, I am thankful SoM for giving me the Graduate Travel Grant to participate an international conference. I would like to express my appreciation to CSI Singapore for furnishing with cutting-edge technologies and well-equipped infrastructures to facilitate cancer biology research. I also appreciated CSI common operation team for preparing routine laboratory supply and maintenance of instruments. My gratitude extended to Department of Pharmacology for administrative assistance.

My thanks extended to the PEL research group for technical support and assistance. I am grateful to my CG members and friends for always being there with me and our invaluable friendships.

Last but not least, I am indebted to my parents for their love and supports. With their encouragement, I have the strength to overcome all the hurdles during these years. I am also grateful to my siblings for their understanding and patience.

Table of Contents

Summary

Angiogenesis is associated with metastasis of mammary carcinoma. Angiogenesis occurs in response to the stimulation of angiogenic factors secreted by mammary carcinoma or stromal cells (Folkman *et al.*, 1971, Folkman, 1992) . Trefoil Factor 3 (TFF3) is a small secreted protein and physiologically involved in mucosal repair and restitution (Taupin and Podolsky, 2003). Increased TFF3 expression is oncogenic and stimulates invasiveness and metastasis of mammary carcinoma cells (Kannan *et al.*, 2010, Pandey *et al.*, 2014).

The role of TFF3 in *de novo* angiogenesis of mammary carcinoma has not been determined. Herein, I defined the effect of TFF3 secreted from mammary carcinoma cells on human umbilical vein endothelial cells (HUVEC) using an indirect co-culture transwell system in which mammary carcinoma cells were plated in the membrane of transwell insert co-cultured with HUVEC seeded in the bottom well of companion plate. Forced expression of TFF3 in mammary carcinoma cells promoted HUVEC monolayer proliferation, cell cycle progression, survival, migration, invasion, and tubule formation *in vitro*. In xenograft models, mammary carcinoma cells with forced expression of TFF3 produced tumors with increased IL-8 expression and enhanced microvessel density (CD31 and CD34 positive) as compared to tumors formed by control cells. Depletion of TFF3 in mammary carcinoma cells by siRNA decreased the angiogenic behaviors of HUVEC. Mechanistically, forced expression of TFF3 in mammary carcinoma cells stimulated IL-8 promoter activity and subsequently increased IL-8 expression. Depletion of IL-8 in mammary carcinoma cells by siRNA or inhibition of IL-8 with anti-IL-8 monoclonal antibody abrogated the stability of TFF3 on stimulation of HUVEC migration, invasion, and tubule formation *in vitro*. Blocking of IL-8 receptor namely CXCR2 in HUVEC by anti-CXCR2 monoclonal antibody inhibited TFF3-stimulated HUVEC tubule formation *in vitro* mediated by IL-8. TFF3 enhanced STAT3 phosphorylation. Depletion of STAT3 in mammary carcinoma cells by siRNA partially abrogated the effect of TFF3 on IL-8 promoter activity and subsequently decreased IL-8 expression. Depletion of STAT3 in mammary carcinoma cells by siRNA also partially abrogated the stimulatory

effects of TFF3 on HUVEC migration, invasion and tubule formation *in vitro*. Furthermore, exogenous rhTFF3 stimulated HUVEC monolayer proliferation, migration, invasion, and tubule formation *in vitro* in a concentration-dependent manner. Exogenous rhTFF3 directly acted on HUVEC to promote angiogenic behaviors of HUVEC. Additionally, TFF3 secreted from HUVEC increased monolayer cell proliferation, cell cycle progression, survival, migration, invasion, and tubule formation *in vitro*. TFF3 secreted from HUVEC enhanced IL-8 promoter activity and increased IL-8 expression in endothelial cells, albeit slightly. Hence, TFF3 is a promoter of angiogenesis, which may co-coordinate with the growth promoting and metastatic actions of TFF3 in mammary carcinoma to enhance tumor progression.

List of Tables

List of Figures

List of Abbreviations

ADH: Atypical hyperplasia
ANGPT1: Angiopoietin 1
ANGPT2: Angiopoietin 2
BrdU: 5-bromo-2-deoxyuridine
CAF: Cancer Associated Fibroblast
CAM: Chorioallantoic membrane
CNS: Central Nervous System
COL18A1: Collagen alpha-1(XVIII) chain
c-Src: Cellular sarcoma
DCIS: Ductal Carcinoma *In situ*
EC: Endothelial Cells
ECM: Extracellular Matrix
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
EMT: Epithelial Mesenchymal Transition
ER: Estrogen Receptor
GI tract: Gastrointestinal tract
GH: Growth Hormone
HER-2/neu: Human Epithelial Receptor 2
hGH: human Growth Hormone
hSIE: human sis-inducible element
HIF: Hypoxia Inducible Factor
hTERT: human Telomerase Reverse Transcriptase
HUVEC: Human Umbilical Vein Endothelial Cell
IBC: Invasive Breast Cancer
IDC: Invasive Ductal Carcinoma
IGF-1: Insulin Growth Factor 1
ILC: Invasive Lobular Carcinoma
LCIS: Lobular Carcinoma *In situ*
MMP: matrix metalloproteinases
NFκB: Nuclear factor kappa B
PDGF: Platelet-Derived Growth Factor
PR: Progesterone Receptor
PI3K: Phosphoinositide 3-kinases
PlGF: Placental Growth Factor
PG: Progesterone
SAGA: Serial Analysis of Gene Expression
STAT: Signal Transducer and Activating of Transcription
TEK: Angiopoietin-1 receptor
TFF: Trefoil Factor
TGFB: Transforming Growth factor beta
TME: Tumor microenvironment
TKI: Tyrosine Kinase Inhibitor
TNF: Tumor necrosis factor
Tsp: Thrombospondin
VBM: Vascular basement membrane
VEGF-A: Vascular Endothelial Growth Factor-A
VEGFR: Vascular Endothelial Growth Factor Receptor

CHAPTER 1

Introduction

Trefoil factors (TFF) family consists of three members, namely TFF1, TFF2, and TFF3. In the normal physiology, all three TFF proteins are expressed in the epithelial cells that line the mucous membrane. The biological functions of TFF peptides include protection of gastrointestinal (GI) tract from injury and mucosal restitution (Taupin *et al.*, 2000b, Taupin and Podolsky, 2003). However, TFF peptides, particularly Trefoil factor 3 (TFF3) have been documented to be over-expressed in human malignancies including breast (Poulsom *et al.*, 1997), gastric (Yamachika *et al.*, 2002, Dhar *et al.*, 2005), prostate (Faith *et al.*, 2004), hepatocellular (Okada *et al.*, 2005), and endometrial (Bignotti *et al.*, 2008). Accumulating evidence suggests that increased expression of TFF peptides is tightly linked to the progression of cancers (May and Westley, 1997a). This introduction will be divided into five sections. The first section describes hallmarks and the development of cancer. The second sections describes pathogenesis and treatment of human mammary carcinoma. The third section describes the biology of TFF3, the signaling pathways of TFF3, and the potential oncogenic effects of TFF3 in cancer progression. The fourth section describes the mechanism and regulation of angiogenesis, and the angiogenic factors involved in tumor angiogenesis. The last section describes the rationales and objectives of this research project.

1.1 Epidemiology of cancer

Cancer is a major disease worldwide with an annual incidence of greater than 10 million (Stewart et al., 2003). In 2012, the newly diagnosed cancer cases and cancer death were approximately 14.1 million and 8.2 million respectively (Ferlay *et al.*, 2012). Cancer is the second commonest of death that accounts with nearly 25% of all deaths. In 2014, the number of newly diagnosed cancer cases is expected to be approximately 1.7 million with cancer deaths of 585,720 in the United States (American Cancer Society, 2014). Current

statistics indicate that 50% of men and 30% of women will develop cancer during their lifetime (Howlader *et al.*, 2014). According to the American Cancer Society, the leading cancer death for men (28%) and women (26%) is lung cancer, followed by prostate (10%) in men and breast (15%) in women (American Cancer Society, 2014). The mortality rate showed only a slight decline, despite advances in early diagnosis and introduction of new therapeutic modalities tailored to target cancer cells. The statistics underlie the importance of understanding the biology of cancer and its signaling pathway.

1.1.1 Heterogeneity of cancer

Cancer is a neoplastic disease characterized by alterations of the genome that results in deregulation of normal cell processes including cell division and cell differentiation. Consequently, the normal balance between the cell replication and cell death is disturbed, thereby, resulting in uncontrolled cell proliferation (Vogelstein and Kinzler, 2004, Ruddon, 2007). Under normal physiological conditions, normal cells divide in response to growth factor signals cease dividing in response to anti-growth signals (Vogelstein and Kinzler, 2004, Ruddon, 2007). The signaling molecules that moderate cell division are released by neighboring cells. In response to genetic or environmental insults, normal cells undergo programmed cell death or apoptosis. Cancer is a genetic disease that originates from a single ancestral cell and is clonal in nature. Tumors starts when a single cell acquires a series of mutations which collectively transforms normal cells into cancerous cells that divide uncontrollably and eventually spread throughout the body (Hanahan and Weinberg, 2000). As cancer growth progresses, genetic drift such as gene alteration or mutation produces heterogeneity in cancer cell population (Futreal *et al.*, 2004). Tumors are classified as either benign or malignant. Benign tumors are non-cancerous cells that unable to invade neighboring tissues or metastasize, and can only grow locally. Malignant tumors are cancer cells that are able to invade, spread to lymph nodes or bloodstream, and metastasize to secondary site in the body (Silverstein *et al.*, 2006). In general, malignant tumors cause

significant morbidity and are fatal if untreated. The etiology of cancer is complex, and partially understood. Current studies indicated that only 5 - 10% of cancer is attributed to genetic defects and the remaining is caused by environmental and lifestyle factors (Anand *et al.*, 2008, Khan *et al.*, 2010), suggesting that cancer is a preventable disease and could be treated if it is detected early with therapeutic treatments.

1.1.2 The hallmarks of cancer

Cancer is a complex and multifactorial disease in which there is strong interplay between genetic and environmental factors. Perturbations of the normal control mechanism drive normal cells to become cancerous cells. Cancer cells acquire a series of genetic mutations that lead to uncontrolled cell growth and division, inhibition of cell differentiation, and resistance to cell death (Futreal *et al.*, 2004). Tumors secrete angiogenic factors that stimulate new blood vessels formation in a process known as angiogenesis to supply cancer cells with oxygen and nutrients, a process called angiogenesis (Folkman, 1990). Some malignant tumors may remain localized and encapsulated. However, most cancer cells eventually invade surrounding tissue and breaking through the basal laminas to spread to distant organs in a process called metastasis (Varmus and Weinberg, 1993). The ability of cancer cells to override normal control mechanisms is underlined by six "hallmarks of cancer" as proposed by Hanahan and Weinberg (2000). The hallmarks of cancer encompass six biological features acquired during cancer cells development. The acquired biological capabilities are as follows (Figure 1):

(i) Self-sufficiency in growth signal.

The ability of cancer cells to divide without growth factor stimulation. Deregulation of cell surface receptors (i.e. growth factor receptors) that rely on growth-stimulatory signals into the cells during tumor pathogenesis and receptors over-expression may also enable the cancer cells to become hyper-responsiveness to growth factors (Fedi *et al.*, 2000). Mutation or structural alterations in cell surface receptors resulted in ligand independent growth signaling

pathways or cross-talking connections with other pathways to trigger multiple biological effects.

(ii) Insensitivity to anti-growth signals.

In cancer, cells acquire the ability to divide in the presence of anti-growth signals. On the contrary, under normal cellular condition, anti-growth signals can inhibit proliferation by forcing actively proliferating cells to enter quiescent (G_0) state. Alternatively, cells may be induced to enter into postmitotic state resulting in inhibition of their proliferative potential. However, cancer cells evade anti-growth signals by avoiding differentiation and promoting growth.

(iii) Evading apoptosis.

Cancer cells acquired capability to escape apoptosis or programmed cell death. The growth of tumor cell is influenced by both the rate of cell proliferation as well as the rate of apoptosis. Acquiring the capability to resist apoptosis can be through a variety of strategies such as mutation in p53 tumor suppressor gene and loss of function of a pro-apoptotic regulator, which resulting in production of functional inactivation of p53 protein (Harris, 1996). An estimated of more than 50% of cancers contained p53 mutations that results in promoting survival signaling pathway instead of apoptotic machinery.

(iv) Limitless replication potential.

Cancer cells acquired capability for unlimited replication and maintenance of telomere length despite repeated cell divisions. Normal cells have a limited number of doublings to be completed before entering senescence (aging process) (Hayflick, 1997). However, cancer cells acquired the capability to multiply without limit and attribute an infinite replicative potential, the trait termed as immortalization, i.e. the ability of a cell to continue dividing without limit. Telomeres are eroded with every cell cycle and the erosion signals a cell to enter senescence. Hence, maintenance of telomerase is observed in 85 - 90% of all type of cancer cells through up-regulation of telomerase enzyme expression or elevating telomerase activity (Shay and Bacchetti, 1997).

(v) Sustained angiogenesis.

Angiogenesis is a cellular event whereby new blood vessels are formed to supply oxygen and nutrients to tissues. It is a tightly regulated process, which becomes deregulated in cancer, whereby neoplastic lesions stimulate sprouting of pre-existing blood vessels or formation of new vasculatures to facilitate tumor growth and metastasis (Bouck *et al.*, 1996, Hanahan and Folkman, 1996).

(vi) Tissue invasion and metastasis.

As cancer progresses, cells acquire capability that enables invasion into surrounding tissues and subsequent dissemination to other parts of the body. The main cause of cancer deaths is due to the metastasis where primary tumor spread to the other parts of the body (Sporn, 1996). Metastasis is a complex mechanism involving dissolution of cell-to-cell contacts (e.g., down-regulation of adhesion proteins such as E-cadherin), alteration of cell-to-microenvironment interactions in cancer through changes in integrin expression, and activation of extracellular proteases and matrix degrading proteases responsible for degrading extracellular matrix (ECM) substrates in cancer cells (Coussens and Werb, 1996, Christofori and Semb, 1999). The capability for cancer cells to invade and metastasize enables escape from the primary tumor site and colonizes at distant organs with sufficient supply of nutrient and growth factors.

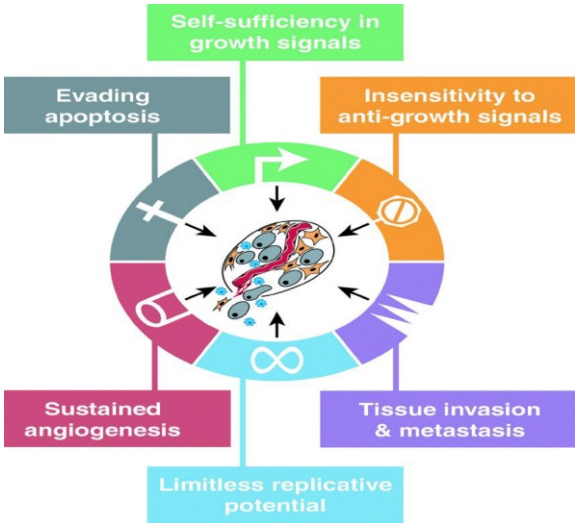


Figure 1: The hallmarks of cancer. The hallmarks of cancer describe functional characteristics, which most cancers acquire during the process of tumor growth and progression (Hanahan and Weinberg, 2000). Reproduced with permission.

The hallmarks of cancer activities are important acquired capabilities that required for development and maintenance of cancer. Further, these acquired capabilities restated that cancer as a multistep disease mainly initiated through the perturbation of the cellular regulatory mechanisms that important for normal cell proliferation as well as homeostasis (Hanahan and Weinberg, 2000). In addition to the existing functional characteristics, genome instability in cancer cells produces genetic variability that accelerates their acquisition of capabilities. Besides, tumor-microenvironment interaction may produce inflammatory responses that are advantages to the cancer cells. The high proliferative state and survival of cancer cells produce genomic instability associated with DNA mutations or DNA damage repair (Charames and Bapat, 2003) and chromosomal abnormalities associated with mutations in oncogenes such as TP53. Consequently, the mechanisms controlling cell cycle, telomere length, and telomerase activity are deregulated (Negrini *et al.*, 2010). Furthermore, two emerging hallmarks have recently been described in the pathogenesis of all if not some cancers (Figure 2). Firstly, deregulating cellular energetics such as modification or reprogramming of metabolism is necessary for cancer cells proliferation. Secondly, avoiding immune destruction provided cancer cells the advantage to escape immunological

surveillance, particularly by lymphocytes and natural killer cells (Hanahan and Weinberg, 2011). Acquisition of these hallmarks lead to the generation of mutant cells with an increased oncogenic potential (Floor *et al.*, 2012).

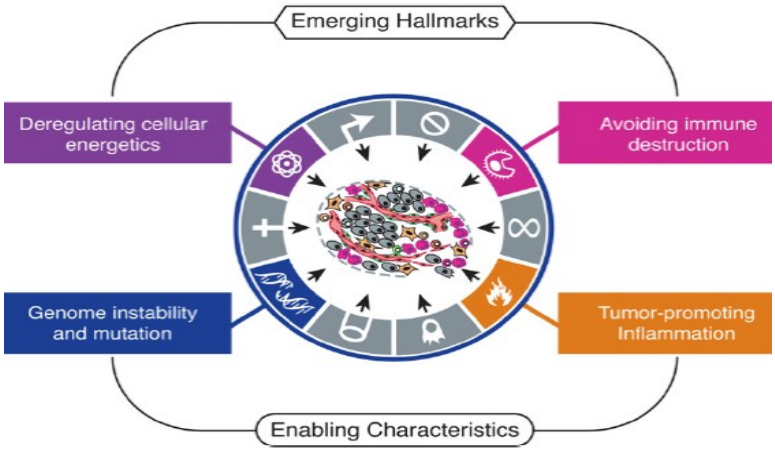


Figure 2: Emerging hallmarks and enabling characteristics. Two additional hallmarks of cancer, namely deregulating cellular energetics and avoiding immune destruction, are implicated in the pathogenesis of cancers. Genomic instability in cancer cells caused by mutations promotes tumor progression. Inflammation initiated by immune cells that function to fight against infections and injury can support multiple hallmark capabilities instead (Hanahan and Weinberg, 2011). Reproduced with permission.

1.1.3 Tumor microenvironment

The highly proliferative cancer cells have been suggested as one of the most crucial agents in the tumor mass. Carcinoma arising from epithelium is initiated by mutation. However, promotion of tumor progression involved the stroma in the tumor environment (Egeblad *et al.*, 2005). Therefore, the series of mutations in cancer cells do not completely elucidate the aggressive or invasive phenotype of tumors (Sager, 1997, Park *et al.*, 2000, Ronnov-Jessen and Bissell, 2009). It was lately discovered that the gene expressions of cells in the tumor microenvironment (TME) is essential for cancer cells to become malignant (Sager, 1997). Emerging evidence indicates that tumorigenesis involve complex heterogeneous interactions between tumors and its interstitium (Liotta and Kohn, 2001, Dvorak *et al.*, 2011). The distinct population of cells that constitute solid tumors include

cancer mesenchymal cells, cancer stem cells, other cell types such as fibroblasts, endothelial cells, stroma cells, and immune inflammatory cells, and the ECM. Together, these components create the tumor microenvironment that contributes to the acquisition of hallmark traits, thereby promoting tumor progression (Hanahan and Weinberg, 2011, Floor *et al.*, 2012).

The capability of the TME to promote tumor growth and progression results from exploiting the ability of its cellular components, particularly cancer-associated fibroblasts, macrophages and endothelial cells (Figure 3). In the immune system, immunological cells play a role to eliminate foreign cellular components such as tumors. However, in cancer, these immunological cells can be paralyzed, which in turn become advantageous for tumor growth and metastasis (Leibovici *et al.*, 2011). The stroma supports tumors growth by providing growth factors from degradation of the extracellular matrix (ECM) by the matrix metalloproteinases (MMPs). Additionally, stroma provides angiogenic factors, which promotes vascularization necessary for growth and metastasis (Leibovici *et al.*, 2011). Therefore, the TME is involved to promote progression of tumor at different stages through the enhancement of invasion, angiogenesis and metastasis (Whiteside, 2008). It has been explicitly shown that tumor microenvironment have a critical part in tumor development and progression (Dvorak *et al.*, 2011).

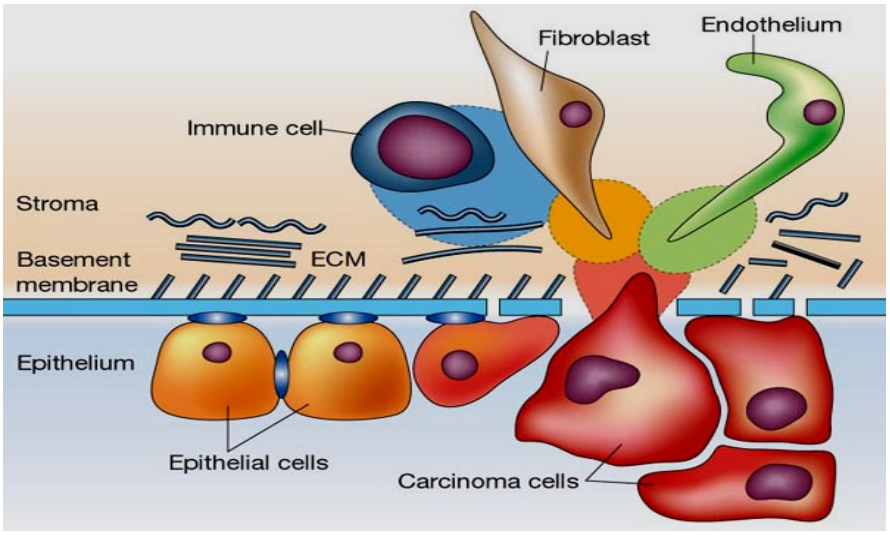


Figure 3: Microenvironment of tumor. The transition from non-invasive to invasive carcinoma required activation of a range of cell types including fibroblasts, immune cells and endothelial cells. Interaction between the stromal cells and the premalignant epithelium is needed for invasion (Liotta and Kohn, 2001). Reproduced with permission.

1.1.4 Invasion metastasis cascade

At advanced stages of primary tumors, cancer cells can gain entry into blood or lymphatic vessels, circulate through the intravascular stream, and proliferate at distant site in the body in a process known as metastasis. The multistep process of cell-biological events has been schematized as sequential and interrelated steps, defined as invasion-metastasis cascade (Fidler, 2003, Talmadge and Fidler, 2010). Each step of this cascade is important as failure to complete any step effectively impedes the tumor cells success to invade and metastasize (Poste and Fidler, 1980). During the process of invasion and metastasis, a series of events have to take place (Figure 4), which begin from local invasion through ECM at primary site, followed by intravasation into blood vessels, survival in circulation, arrest at secondary site, subsequently extravasation into secondary tissues, survival in new microenvironments by forming micrometastases, reinitiation of proliferation, and finally production of macroscopic tumors (referred to "metastatic colonization") (Fidler, 2003, Hanahan and Weinberg, 2011).

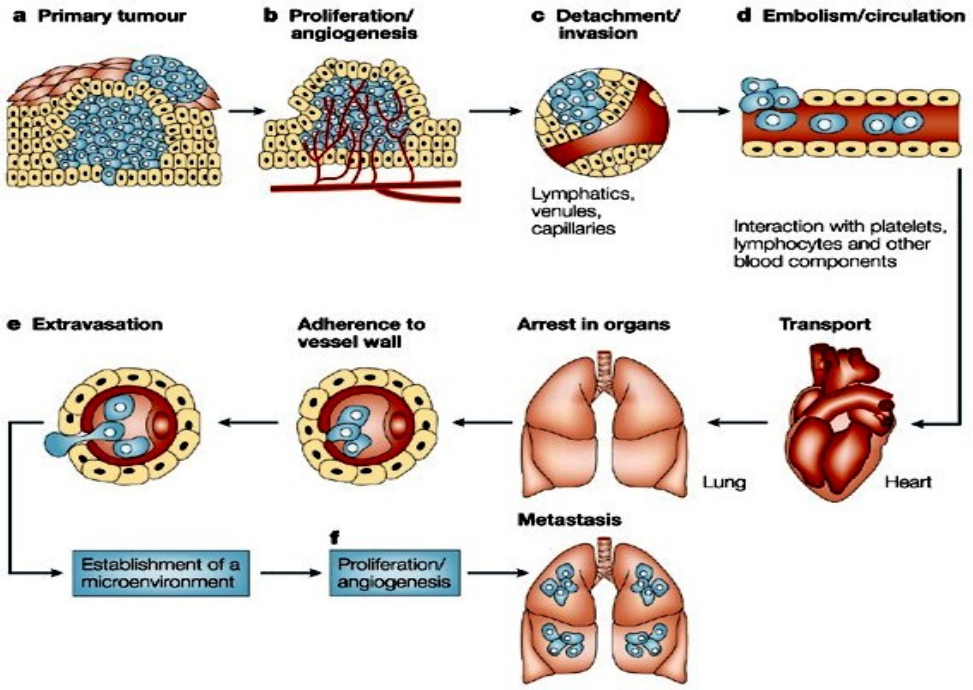


Figure 4: The invasion-metastasis cascade. Invasion-metastasis is a complex series of cellular events. During metastatic progression, cancer cells invade locally from the primary tumor site, break through the basement membrane, enter into the circulation, arrest at a secondary site, and survive in the secondary tissues (Fidler, 2003). Reproduced with permission.

Accumulating evidence have shown that the normal tissue architecture serves as barrier against invasion which cancer cells have to break through to metastasize successfully (Valastyan and Weinberg). The ECM in tissue architecture provides a support for organization of cells into specific tissues. For example, basement membrane of the ECM structures served as physical barrier that prevents invasion and metastasis. To initiate the invasion-metastasis cascade, cell invasion initially participated in modification of cell adhesion, degradation of ECM, and migration of tumor cells. Subsequently, the cancer cells could co-opt with epithelial-mesenchymal transition (EMT) program that implicated in normal embryonic morphogenesis (Shook and Keller, 2003). Epithelial cells are connected with each other by cell-cell junctions to ensure the integrity and the stabilization of the epithelium. During the process of EMT, the tumor cells acquire invasion and migration characteristics, thereby promoting the cancer cells migrate through the ECM (Figure 5).

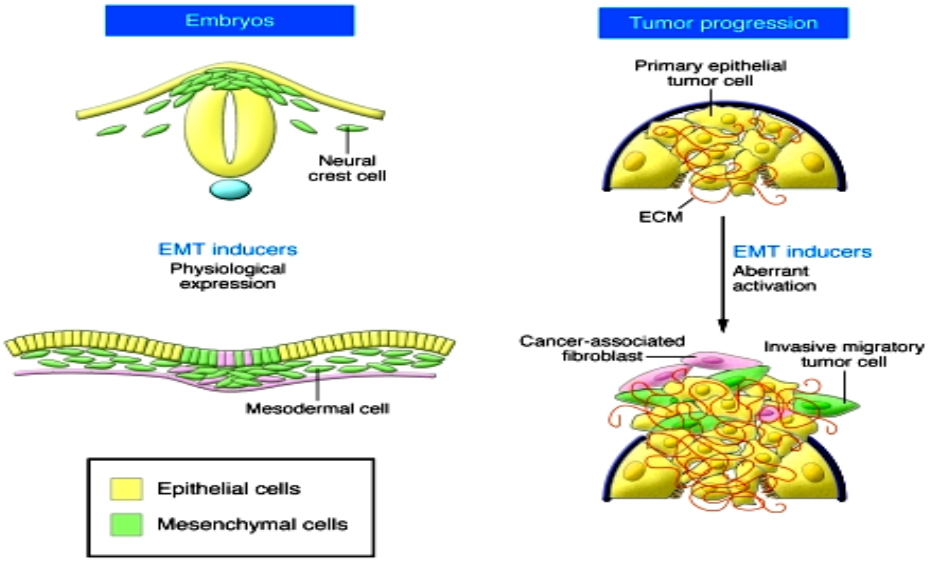


Figure 5: EMT in development and disease. In normal conditions, EMT (cells in green) occurs during development of embryos, such as during neural crest cell delamination from the dorsal neural tube. EMT inducers usually remained quiescent but become highly active in invasive carcinomas. The influence of cell state during development or disease is highly dependent on epithelial-mesenchymal transitions (Acloque *et al.*, 2009). Reproduced with permission.

EMT inducers are activated in primary tumor which lead to the dissociation of adherens or tight junctions, loss of cell polarity, transition of epithelial cell to adopt mesenchymal characteristics which include increased cellular invasiveness and aggressiveness (Baum *et al.*, 2008, Thiery *et al.*, 2009) (Figure 6). Furthermore, EMT is organized by a set of transcription factors, including ZEB1, SLUG, TWIST, SNAIL, and ZEB2 that allows carcinoma cells to gain mesenchymal properties by repressing expression of epithelial markers and inducing expression of mesenchymal markers (Thiery *et al.*, 2009).

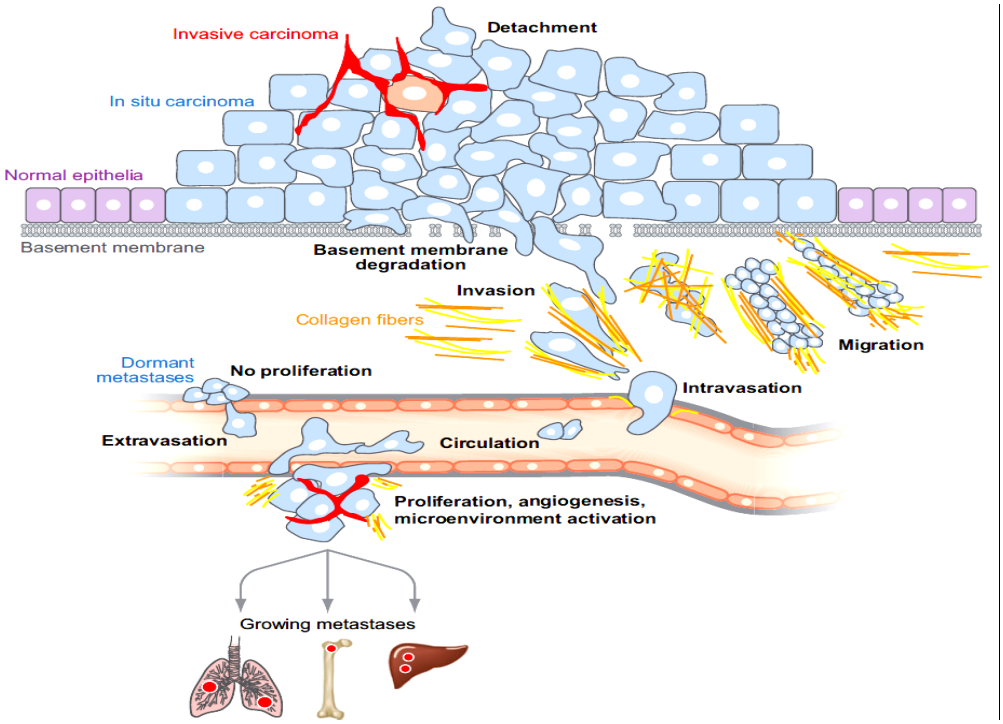


Figure 6: Cellular events in EMT. The EMT occurs at the primary tumor site whereby normal epithelial cells transform into invasive carcinoma *in situ* due to the loss of tight/adherents junctions as well as cell polarity. As basement membrane becomes degraded, tumor cells begin to invade the surrounding stroma to intravasate into blood vessels or lymph node, and finally be transported in circulation until they colonize at a distant organ (Bacac and Stamenkovic, 2008). Reproduced with permission.

EMT is induced by multiple signaling pathways that mediated by growth factors, Src, Ras, Integrin, Wnt/beta-catenin and Notch. One of the most important molecular characteristic of EMT is the down-regulation of E-cadherin, which is a cell adhesion molecule commonly found in normal epithelial cells and plays a role in tumor suppression. However, the expression of E-cadherin is found to be down-regulated during cell transformation (Larue and Bellacosa, 2005). Numerous studies have shown that TGF-β to be a multifunctional peptide can suppress or promote tumors formation (Muraoka-Cook *et al.*, 2005, Lebrun, 2012). The TGF-β pathway is found to suppress cell transformation by preventing cell division and inducing cell death or senescence. During late tumorigenesis, stimulation of TGF-β signaling is associated with cancer-related events such as promoting cell proliferation, cell motility, new blood vessels development, invasion and metastasis (Muraoka-Cook *et al.*, 2005, Lebrun, 2012). Metastatic events are stimulated by EMT through activation of the

phosphatidylinositol 3' kinase (PI3K)/Akt signaling pathway that induces Slug- or Snail-mediated repression of E-cadherin gene, which in turn resulted in alterations of cell morphology, tumorigenicity, cell motility and invasiveness (Larue and Bellacosa, 2005). TWIST is an EMT transcription factor, which induces EMT and contributes metastatic feature of tumors (Yang *et al.*, 2004). Therefore, multiple signaling pathways determine the growth and metastatic potential of tumors.

1.2 Human mammary carcinoma

Human mammary carcinoma is also known as breast cancer. It is a type of tumor that originates from breast tissue i.e. epithelial cell in the inner lining of milk ducts or the lobules (Sariego, 2010). Benign breast conditions (non-cancerous) are very commonly found in women. Non-invasive or *in situ* breast cancers are malignant breast tumors that remain inside the ducts and have not spread into the surrounding breast tissue (Majure, 2000). This type of breast cancer could be cured by early detection and treatment. In contrast, invasive (infiltrating) breast cancers spread beyond the membrane lining a duct or lobule, invade the neighboring tissue and metastasize to the liver, lung, and brain (Majure, 2000). This type of breast cancer can severely affect the mortality or morbidity of women.

1.2.1 Epidemiology of mammary carcinoma

Mammary carcinoma is the major cause of death for women worldwide that accounts for 29% (232,670) of newly diagnosed cancer cases and 15% (40,000) of total cancer mortality in the United States (American Cancer Society, 2014). It is estimated that about 12.3% women will be diagnosed with breast cancer in their lifetime (Siegel *et al.*, 2014). Moreover, World Health Organization (WHO) predicts that up to 70% of the new breast cancer cases will occur in the developing countries by 2020 (Ferlay *et al.*, 2012) and that 84 million of people will be affected in the next 10 years if no actions are taken (Morabia and Abel, 2006)

In Singapore, cancer is the leading cause of mortality, constituting 18,481 of total cancer deaths and 56,316 new cancer cases in 2012. The incidence rates of cancer have increased for the period 2006 - 2010 (Teo and Soo, 2013). Breast cancer is the top frequently diagnosed cancer (29.4%) with mortality rate of 17.9% in Singapore women (Singapore Cancer Registry, 2012). About 1,600 women are diagnosed with breast cancer each year (Singapore Cancer Registry, 2012). GLOBOCAN 2012 revealed a sharp rise of breast cancer incidence in women and highlighted to the importance and priority of preventive and control measures (Ferlay *et al.*, 2012). There is an urgent demand for eradicating breast cancer, encouraging considerable efforts to elucidate the underlying mechanism of this deadly disease.

1.2.2 Development and structure of normal human mammary gland

The mammary gland is a highly complex, dynamic, and specialized organ that consists of multiple lobes. Each lobes are made up of several lobules that contain multiple acini, which eventually drain milk into the nipple via the ducts (Owens *et al.*, 2013). The basic functional unit in the human mammary gland is terminal end bud (TEB) consists of a lobule and the draining duct supported by a network of adipose tissues and connective tissue (Ramsay *et al.*, 2005). Human mammary gland is composed of glandular structures whose major function is to produce milk under the influence and control of hormones.

Mammary gland development take places at different stages such as embryonic, prepubertal, pubertal, pregnancy, lactation, and involution that are related during reproduction as well as sexual development (Hennighausen and Robinson, 2001). In the embryogenesis of mammary development, both males and females share the same primitive mammary gland at birth. In female, mammary gland development is initiated during the onset of puberty and is regulated by the elevated secretion of ovarian hormones such as estrogen and progesterone. After puberty, the female mammary gland experienced cycles of growth and morphologic changes including TEB formation, ductal elongation, lateral branching, and terminal duct lobular formation as well as stroma expansion. Additionally, morphologic changes to or

regulation of female mammary glands are governed by the menstrual cycle, pregnancy and lactation (Ali and Coombes, 2002). Unlike females, the male mammary glands do not undergo morphologic changes and remained in quiescent state (Sternlicht, 2006). The female mammary gland consist a primitive branching ductal system and the process of duct and acini development is known as branching morphogenesis. The duct originates in the fetus and terminates at early childhood until puberty when hormones begin to stimulate and trigger differentiation. Under hormonal influences, the interaction between epithelium and mesenchyme results in differentiation of the prenatal primitive structure to produce a mature mammary gland (Javed and Lteif, 2013).

Humans have two complex mammary glands that consist of 10 - 20 simple glands in each breast. Two tissue compartments that constitute the mammary gland are the parenchyma, which forms the branching duct system from which secretory acini develop. The mammary fat pad is made up of adipose stroma or connective tissue which serves a support in which the parenchyma develops and functions (Medina, 1996). The parenchyma is composed of the alveoli that are made up of milk-secreting epithelial cells that are surrounded by myoepithelial cells (Figure 7). The branching ductal system is mainly developed during puberty. In contrasts, the lobuloalveolar begins to develop during pregnancy. The ducts branch into smaller ductules that terminate in lobules.

The mammary gland is composed of approximately 15 - 20 lobes. The mammary epithelium is mainly composed of two cell types, the basal and the luminal. The basal epithelium comprises the myoepithelial and a subpopulation of stem cells. The myoepithelial cells possess contractile functions and form the outer layer of the mammary gland. The function of the small subpopulation of stem cells is to generate or produce different cell types. The luminal epithelium gives rise to the ducts and secretory alveoli. Both myoepithelium and luminal epithelium function together to generate a bi-layered, tubular structure that enables contraction of myoepithelial cells to discharge milk during lactation (Macias and Hinck, 2012).

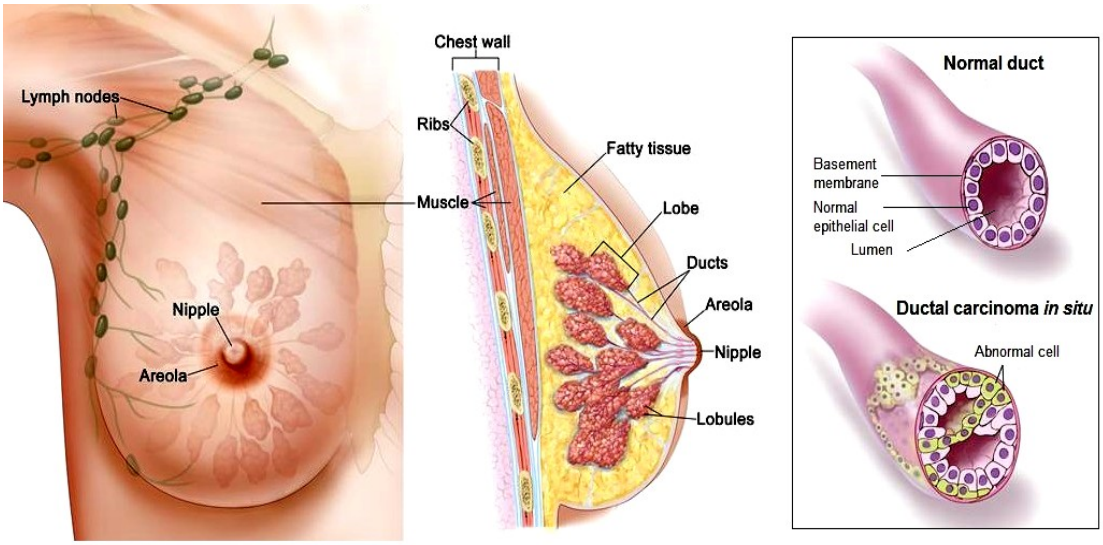


Figure 7: Anatomy of the human mammary gland. Mammary carcinoma duct is covered with a layer of epithelial cells that function in producing milk. The glands are surrounded by an outer layer of myoepithelial cells that possess contractile properties. Abnormal cells are found in ductal carcinoma. Adapted from http://www.breastcancer.org.

It has been proposed that the cell lineages that compose the mammary gland are derived from a mammary stem cell population (Smalley and Ashworth, 2003). A hierarchy of stem cells including unipotent and multipotent cells appear to be present within the mammary gland whereby each of them likely to have a distinct function in the morphogenesis and mammary epithelium maintenance (Van Keymeulen *et al.*, 2011). These cells are capable of differentiating into multiple cell type of the mammary gland depending on different stages such as during development, pregnancy or even self-renewal. Self-renewal is essential to drive the growth of subsequent pregnancies due to the massive apoptotic events occurring during post weaning. (Tiede and Kang, 2011). It has been postulated that the mammary stem cells (MSCs) made up the epithelial compartment of the mammary gland. These MSCs possess self-renewal potential and produce epithelial precursor cells, which are further differentiated into either basal or luminal epithelial (Figure 8). The presence of stem cells is the fundamental for renewal of alveolar in each pregnancy.

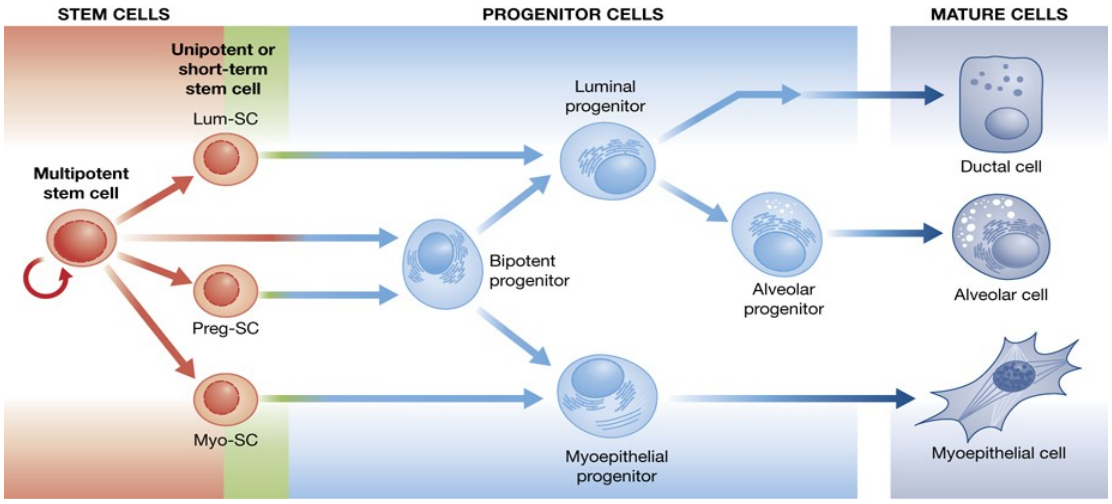


Figure 8: Model of the epithelial differentiation hierarchy in the mammary gland. A heterogeneous compartment of stem cells is present in mammary gland, in which the multipotent stem cell has greater self-renewal capacity than the unipotent stem cells (Myo-SC: myoepithelial-stem cell, Lum-SC: luminal-stem cell, Preg-SC: short term pregnancy-stem cell). The bipotent progenitors develop into either luminal or myoepithelial progenitor. Myoepithelial cells and luminal cells are formed as the ducts grow out postnatally during puberty. On initiation of pregnancy, myoepithelial and luminal cells are generated to synthesize and secrete milk (Visvader and Lindeman, 2011). Reproduced with permission.

1.2.3 Development and hormonal control of the mammary gland

At puberty and pregnancy stages, the mammary gland undergoes tissue remodeling in response to hormonal stimuli (Richert *et al.*, 2000, Hovey *et al.*, 2002). During puberty, estrogen and growth hormone function together to initiate mammary duct elongation. During pregnancy, other hormones such as progesterone (Pg) and prolactin (PRL) work coordinately to stimulate alveolar formation for production of milk for the young. Morphologic changes to the mammary gland are influenced by the presence and secretion level of hormones through paracrine actions and by transcription factors that modulate mammary stem cells activity (Asselin-Labat *et al.*, 2006, Joshi *et al.*, 2010). During the post-pregnancy stage, mammary epithelial cells express estrogen and progesterone receptors to enable ductal elongation, branching and alveolar expansion through regulating cell proliferation and turnover of cells in estrous cycles. Both estrogen and progesterone also function in other organs such as the uterus, ovaries and the hypothalamus for regulation of sexual development (Hennighausen and Robinson, 2001).

Mammary gland development is controlled by a variety of steroid hormones and their receptors. Estrogen binds to two distinct estrogen receptors, ERα and ERβ, which functions as transcription factors when binding of steroid hormone. ERα plays a role during normal ductal outgrowth and elongation during puberty (Bocchinfuso *et al.*, 2000). On the contrary, ERβ deletion does not have significant effects on the development of alveolar or duct (Forster *et al.*, 2002). Progesterone and its cognate receptors (A and B isoforms) play a crucial function during pregnancy stage by inducing mammary ductal side branching essential for lobuloalveolar development (Brisken *et al.*, 1998, Atwood *et al.*, 2000). Progesterone receptor B (PR-B) primarily mediates progesterone proliferative effects during pregnancy (Bromberg and Darnell, 2000). Prolactin (PRL) is a protein hormone produced by the pituitary gland that stimulates mammary gland development and milk production (Bole-Feysot *et al.*, 1998). PRL has a direct function on the formation of alveolar bud, however it does not have any contributions in ductal side branching in adult mammary glands (Ormandy *et al.*, 1997). Prolactin (PRL), cooperate with cortisol and insulin to stimulate transcription of the genes that encode milk proteins (Ormandy *et al.*, 2003).

1.2.4 Risk factors and etiology of mammary carcinoma

The incidence rate of breast cancer is determined by sexual reproduction, lifestyle, age, and many other factors (Key *et al.*, 2001). The breast cancer risk is greater amongst women with early age at menarche, delayed age at first full term pregnancy, breast feeding duration and late age at menopause, all of which are associated with prolonged exposure to estrogen (Lester, 2010). Collaborative Group on Hormonal Factors in Breast Cancer (Beral and Collaborative Group on Hormonal Factors in Breast, 1997) reported that the use of oral contraceptives and postmenopausal hormonal replacement therapy increase the risk of this disease as these exogenous estrogen agents increase cancer cells proliferation and induce mutagenesis (Persson, 2000). More than 60% of all breast cancers are dependent on hormones such as estrogen for their growth (Pasqualini, 2004). One of the definite risk factors of breast

cancer is history of atypical hyperplasia, which has a higher prevalence of transforming to malignant tumor than non-atypical proliferative breast lesions. Women with high density breast tissue, that has higher density of epithelial component compared to fatty and connective tissue component, are more likely to develop breast cancer than women with low density breast tissue (McCormack and dos Santos Silva, 2006). Other risk factors correlated to higher risk of breast cancer include increase height (Green *et al.*, 2011), higher body mass index (Reeves *et al.*, 2007), obesity, alcohol consumption and exposure to irradiation (Lester, 2010).

The incidence of breast cancer is also significantly increased in women with family history of breast cancer such as women with one first-degree relative with breast cancer have higher risk as compared with those without family history of the disease (Pharoah *et al.*, 1997). Furthermore, hereditary breast cancer accounts for close to 15% of cases and is caused by germline mutations in cancer susceptibility genes such as *BRCA1*, *BRCA2* and *TP53* (Thull and Vogel, 2004, Monnerat *et al.*, 2007). BRAC1 and BRCA2 proteins are important in maintaining genomic stability for homologous recombination and DNA repair of normal cells (Gudmundsdottir and Ashworth, 2006). Genetic mutation in *BRCA1* and *BRCA2* are associated with familial breast cancers. Individuals inherit these gene mutations have a range of 50% - 87% lifetime risk of development of breast cancer. Loss of function of such as tumor suppressor genes can result in high grade tumor and breast cancer progression (Osborne *et al.*, 2004). The p53 tumor suppressor protein regulates genes involved in cellular processes including apoptosis, cell cycle, and senescence (Brosh and Rotter, 2009). It has been reported that over-expression of mutated p53 protein in breast cancer patient is associated with poor prognosis and a greater incidence of recurrence (Macdonald, 2004). Additionally, over-expression of HER2/neu, a transmembrane tyrosine kinase member of the epidermal growth factor receptor family, was observed in 30% patients with breast cancer. Several studies have concluded that over-expression of this receptor is associated with a poor survival outcome of breast cancer patients (Ross and Fletcher, 1998, Nunes and Harris, 2002).

Nevertheless, the beneficial factors such as healthy lifestyle, physical exercise, lower age of first pregnancy, having more children and prolonged breastfeeding is positively associated with a reduced risk of developing breast cancer (Baselga and Norton, 2002).

1.2.5 Development of mammary carcinoma

Breast cancer is a complex and heterogeneous disease that is developed during the process of lesions from epithelial hyperplasia to invasive carcinoma and eventually metastasis (Allred *et al.*, 2001, Polyak, 2007). Breast cancer is initiated from a series of transformation events (genetic or epigenetic) in a single cell. The subsequent tumor progression is the resultant of accumulation of various genetic alterations such as amplification of oncogenes and/or mutation/loss of tumor suppressor genes (Beckmann *et al.*, 1997). A study by Allred *et al.* (2001) has demonstrated that the development of breast cancer is a multistage progression involving series of intermediate hyperplastic lesions such as epithelial hyperplasia, atypical ductal hyperplasia (ADH), followed by growth into non-invasive *in situ* carcinoma (ductal carcinoma *in situ* or lobular carcinoma *in situ*), invasive carcinoma, and finally metastatic cancer (Allred *et al.*, 2001). Normal breast duct consists of an epithelial cells layer and a myoepithelial cells layer that are separated from the stroma by a basement membrane. The ducts are surrounded by an interstitium consists of extracellular matrix (ECM) and stromal cells (e.g., endothelial cells, fibroblasts and leukocytes) (Man and Sang, 2004, Man, 2007). ADH is a premalignant lesion characterized by the presence of abnormal cell layers within the duct or lobule. The epithelial cells in ADH are abnormal in number, size, shape, appearance, and even excessive growth of cells in the ducts or lobules. ADH triggered by perturbed hormonal levels, inflammatory response and neoplasia. ADH increases the lifetime risk for developing of breast cancer (Vargo-Gogola and Rosen, 2007). *In situ* carcinomas are composed of lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS). DCIS is a non-invasive cancer in which abnormal outgrowth of cells are confined to the lumen of the mammary duct. LCIS is an abnormal outgrowth of cancer cells in the milk-

producing lobules. The presence of DCIS can greatly increase the risk of developing into invasive breast cancer (Khamis *et al.*, 2012) as it is a precursor lesion of invasive growth called invasive breast cancer (IBC) (Figure 9). Unlike DCIS, LCIS is not a precursor lesion for breast cancer (Rieger-Christ *et al.*, 2001). The risk of developing invasive and/or malignant breast cancer increases with each stage. There are mainly two types of IBC, the invasive ductal carcinoma (IDC) and the invasive lobular carcinoma (ILC). IDC is the most common invasive mammary carcinoma (70% of cases) as compared to ILC, which represents about 10% of all breast cancer cases. Advanced mammary carcinoma is associated with degradation of basement membrane and disruption of the mammary myoepithelial cells. Tumor cells invade neighboring tissues, spread to distant organs, and subsequently metastases may be occurred via bloodstream or lymph nodes and subsequently led to dissemination of tumor cells to distant organs such as the bone, lung, liver, and less frequently the central nervous system (Solomayer *et al.*, 2000, Mack *et al.*, 2004).

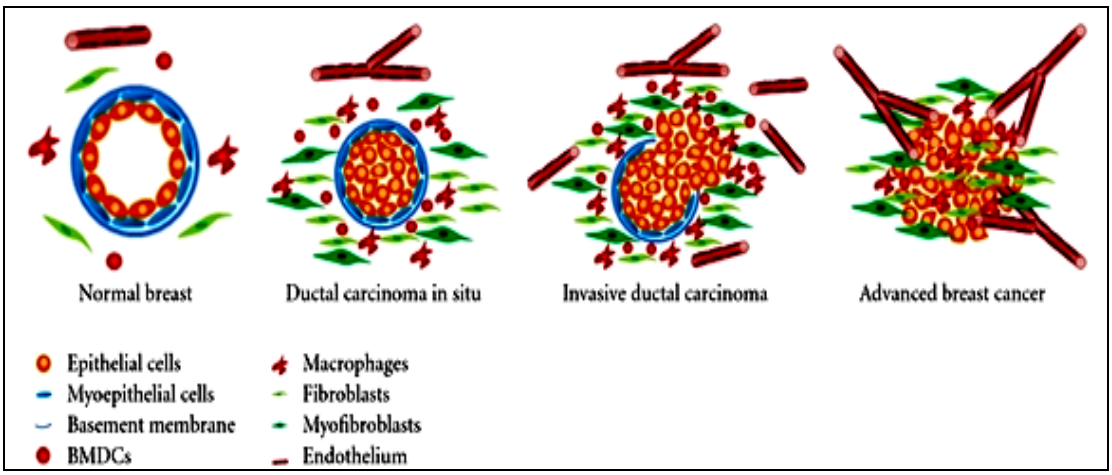


Figure 9: Breast cancer progression. Normal breast duct is composed of a layer of epithelial cells and a layer of myoepithelial cells. Ductal carcinoma *in situ* (DCIS) is an abnormal outgrowth of carcinoma cells confined within the ducts and lobules of the breast. When the myoepithelial cell layer and basement membrane are completely lost, carcinoma cells escape from the barrier of ducts or lobules, invade the surrounding breast tissues, and becomes invasive mammary carcinoma (Khamis *et al.*, 2012).

1.2.6 Microenvironment of mammary carcinoma

The microenvironment of mammary carcinoma plays an essential role in the control of neoplastic progression through dynamic reciprocity between cancer cells and their surrounding environment or tissues (Polyak and Kalluri, 2010). The mammary duct consists of epithelial cells surrounded by stroma components includes fibroblasts, myofibroblasts, endothelial cells, adipocytes, and various immune cells (Erler and Weaver, 2009). A thin layer of ECM lies between the epithelial cells and the stroma (Woodward *et al.*, 1998). The ECM is a rich in matrix metalloproteinases (MMPs) and growth factors that participate in neoplastic dissemination (Finger and Giaccia, 2010). There are four different types of cellular connections essential in the maintenance of the epithelial layer, which include desmosomes, adherens junctions, gap junctions, and tight junctions (Ehmann *et al.*, 1998). The functions of these structures are to restrict both cell movement and unlimited proliferation. Gap junctions act as a channel to mediate cell-to-cell communication and channeling cellular biological signals such as cytokines or products of tumor suppressor genes to the neighboring cells. Under normal physiological conditions, proper tissue architecture is preserved and/or retained by basement membrane and intercellular communication (Joyce and Pollard, 2009).

The normal tissue microenvironment acts as a barrier to tumorigenesis and exerts protective constraint against malignant transformation (Figure 10). The microenvironment can act as a permissive forces to tumor growth in the process of mammary carcinoma progression (Bissell and Hines, 2011). During invasion, tumor cells induce disruption of cell-cell contacts and cell-ECM interactions mediated by cell adhesion molecules such as integrins and cadherins. The basement membrane undergoes degradation and structural changes that lead to the destruction of normal tissue boundaries and provide a path for invasion of cancer cells (Khamis *et al.*, 2012). Therefore, the metastasis of mammary carcinoma is promoted by proteolytic degradation of neighboring tissue while motility of cancer cells is enhanced by the actions of chemokines, growth factors and also recruitment of stromal cells. (Friedl and Wolf, 2003). Several studies have demonstrated that cancer-associated fibroblasts stimulated

motility and proliferation of cancerous epithelial cells when they had direct cell interaction in co-culture (Korohoda and Madeja, 1997, Olumi *et al.*, 1998, Kalluri and Zeisberg, 2006). Endothelial cells are also recruited to the tumor microenvironment for ectopic blood vessels formation for metastasis (Folkman, 1992). The cellular transformation is regulated by interaction between epithelial cells, activated stromal cells as well as the ECM components (Khamis *et al.*, 2012). These cellular components in tumor microenvironment may function either as suppressive or promotional forces in the progression of mammary carcinoma (Bissell and Hines, 2011).

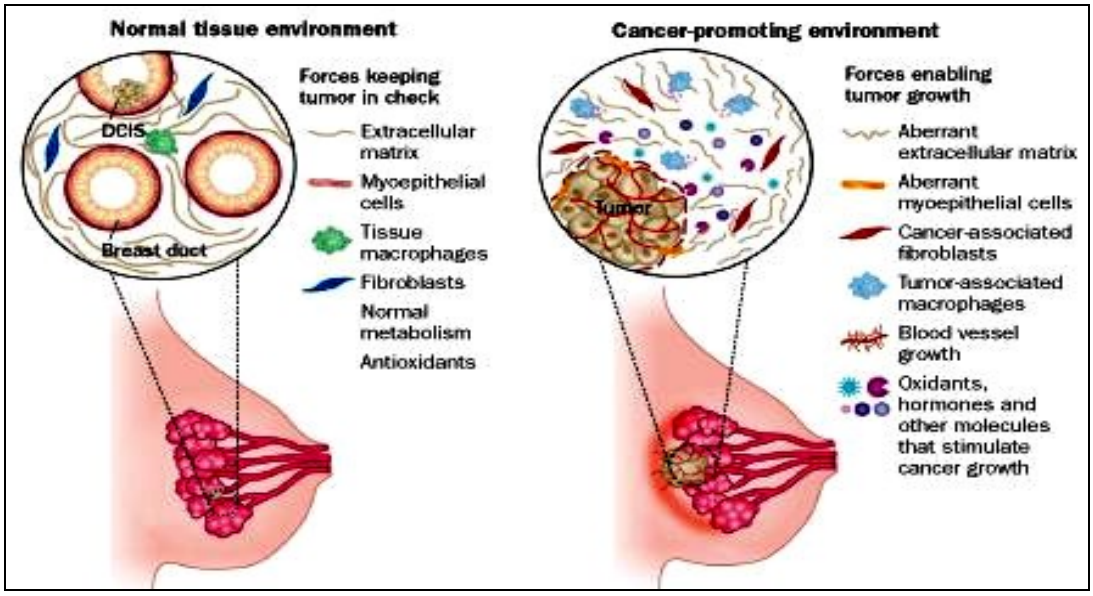


Figure 10: Microenvironment of breast cancer development. In a healthy breast (left), the normal tissue microenvironment acts as a barrier to tumorigenesis and exerts suppressive forces to restrain the dormant tumors under normal tissue homeostasis. In invasive cancer (right), the microenvironment can turn to be permissive conditions for tumor growth, and the combination of mutagens, inflammation, growth factors and other tissue-associated promotional forces can breach the barrier to tumor formation, resulting in aggressive growth of cancer (Bissell and Hines, 2011). Reproduced with permission.

1.2.7 Oncogenes in pathogenesis of mammary carcinoma

Pathogenesis of mammary carcinoma is associated with the accumulation of alterations of the genome. Genetic alterations that repress tumor suppressor genes and activate oncogenes may prime the affected cancer cells to be more susceptible to gain additional genetic damage that eventually led to instability in the tumor genome (Al-Kuraya *et al.*, 2004). Increased genetic alterations have been associated with clinicopathological features such as poorly differentiated pathology, metastasis and decreased survival (Al-Kuraya *et al.*, 2004, Ellsworth *et al.*, 2008a). Several oncogenes have been linked to mammary carcinoma progression including *ER*, *PR*, *HER-2/neu* (*ErbB-2*), and *c-myc*. These genes are the major drivers of cancer progression and therefore become targets for therapeutic intervention.

(i) Estrogen receptor

The ER is one member of the nuclear hormone family of intracellular receptors and is activated upon binding of its hormone ligand, 17β-estradiol (Dahlman-Wright *et al.*, 2006). ER functions as a DNA-binding transcription factor that regulates gene expression (Levin 2005). There are two main isoforms of ER namely ERα and ERβ that encoded by *ESR1* and *ESR2* genes, respectively. In response to the stimulation of estrogen, ERs dimerize to form functionally active homodimer or heterodimer which then activate the transcription of the reporter genes containing estrogen response elements (Cowley *et al.*, 1997, Pace *et al.*, 1997). ERs are overly expressed in about 70% of breast cancer cases (ER-positive breast cancer). Upon binding of estrogen, ER induces mammary cells proliferation that subsequently lead to a marked increase in cell division and mutation rate (Burns and Korach, 2012). Furthermore, the process of metabolizing estrogen may produce genotoxic byproducts, which can directly damage DNA and subsequently resulted in point mutations (Burns and Korach, 2012). ERα expression is associated with more differentiated tumors while the significance of ERβ expression in tumor remains controversial (Herynk and Fuqua 2004). One study suggested that ERβ is correlated with cell proliferation and poor prognosis (Rosa *et al.*, 2008).

(ii) Progesterone receptor

The progesterone receptor (PR) is a transcription factor and a member of a group of intracellular steroid hormone receptors, which regulate gene expression at specific sites in DNA (Lange and Yee, 2008). There are two PR isoforms, PR-A (94 kDa) and PR-B (116 kDa), produced by the same gene located on chromosome 11 (Law *et al.*, 1987) that regulate different but overlapping target genes (Graham *et al.*, 2005). Comparing with PR-A, PR-B is a more potent transcriptional activator. PR-B has an effect on cellular proliferation and also differentiation of mammary epithelium. The biological function of ovarian and uterine are dependent primarily on PR-A (Mulac-Jericevic *et al.*, 2003). PR is expressed in reproductive tissue and has crucial roles in folliculogenesis, ovulation, implantation and pregnancy (Gadkar-Sable *et al.*, 2005). Recent clinical studies have shown that progesterone possess proliferative property in the normal human breast that is independent of estrogen, and is also one of the risk factor for mammary carcinoma (Anderson, 2002, Lee *et al.*, 2006). Furthermore, progesterone/PR acts in conjunction with mitogenic protein kinases and cell-cycle mediators to stimulate regulation of PR target genes (Lange and Yee, 2008).

(iii) HER2/neu

The HER2/neu (ErbB-2 or ERBB2) is a member of the epidermal growth factor receptor (EGF-R) family with tyrosine kinase activity and *HER2/neu* gene is found on chromosome 17q12 (Coussens *et al.*, 1985). HER2 is a membrane receptor tyrosine kinase implicated in the signal transduction pathways connected to cell growth and differentiation (Slamon *et al.*, 1989). Presumably, HER2 is an orphan receptor, which do not have a known ligand and is thus not activated by EGF family ligands (Olayioye, 2001). However, HER2 serve as a preferential dimerization partner for other members of the ErbB family (Olayioye, 2001). An estimated of 30% of breast and ovarian cancers contained *HER-2/neu* gene amplification and/or over-expression of its protein (Zhou and Hung, 2003). Over-expression of HER2/neu receptor in breast cancer has been reported to be associated with increased disease recurrence as well as worse prognosis (Ellsworth *et al.*, 2008b). This poor prognosis

may be due to genomic instability where breast cancer cells with higher frequencies of chromosomal modification is associated with enhanced cell proliferation and a more aggressive phenotype (Ellsworth *et al.*, 2008b). Additionally, genetic changes of the proto-oncogene *ErbB-2* (*HER2/neu*) are connected with elevated invasive phenotype and poor prognosis in breast cancer patients (Wilson *et al.*, 2002).

(iv) c-myc

The c-myc protein is a transcription factor and contains basic helix-loop-helix (bHLH) domain (Vennstrom *et al.*, 1982). The *c-myc* oncogene is over-expressed in about 15 - 25% of breast tumors (Deming *et al.*, 2000) and is implicated in breast cancer cell growth, transformation, angiogenesis, as well as cell cycle control (Chen and Olopade, 2008). Numerous studies have consistently observed the positive correlation between amplification of *c-myc* gene with breast cancer progression and poor clinical outcome (Chen and Olopade, 2008). Therefore, *c-myc* gene amplification is identified as a strong prognostic marker in predicting early recurrence in patients with node-negative breast cancer (Schlotter *et al.*, 2003).

1.2.8 Histological and molecular grading systems in mammary carcinoma

Histological-based grading system provides a method to distinguish well-differentiated tumors from poorly differentiated tumors. Grade 1 refers to tumors that are well-differentiated and demonstrates good prognosis, whereas Grade 3 is poorly-differentiated tumor and it is more likely to spread (Elston and Ellis, 1991). Although histological based grading system is widely used in the past, it may not be clinically informative as majority of breast cancer cases are fall under Grade 2 (Sotiriou *et al.*, 2006). The American Joint Committee on Cancer (AJCC) staging system of breast cancer serves is a standard for grouping patients with respect to prognosis. Therapeutic options are chosen based on several factors such as tumor size, lymph node status, hormone receptor status, HER2/neu status, and menopausal status. The AJCC has designated the staging system by

TNM classification to define breast cancer, where T to tumor size, N to lymph node involvement and M to distant metastasis (Singletary *et al.*, 2002). TNM refers to the presence of invasive or non-invasive tumor, the tumor size, the presence of cancer cells in lymph node and the presence of secondary metastasis of the tumor cells. In addition to the clinical TNM staging, current therapeutic options are preferably combined with histopathological grading, tumor size, lymph node, hormone receptor, HER2/neu and proliferation (Ki67) status. Therefore, molecular subtyping with immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) have later been established (Perou *et al.*, 2000). This classification may provide additional information on patient survival outcome. There are four molecular subtypes including luminal-like (luminal A or luminal B), HER2 enriched/over-expression, basal-like (triple negative) and normal-like. According to this classification, ER-negative breast cancer comprises of two tumor subtypes: basal-like and HER2-positive. Recent studies have demonstrated that these molecular subtypes differ in their responses to treatment and survival outcomes (Blows *et al.*, 2010, Dawood *et al.*, 2011). Other studies have precisely classified these molecular subtypes of breast cancer with respect to their PR, ER, and HER2 status and categorized them as luminal B (ER+ or PR+, and HER2+), luminal A (ER+ or PR+ and HER2-), basal (ER-, PR-, and HER2-) and HER2 (ER-, PR-, and HER2+) (Brenton *et al.*, 2005, Carey *et al.*, 2006). The luminal subtypes are the most common breast cancer subtypes and present good prognosis. Conversely, HER2 and basal-like subtypes show poor prognosis (Brenton *et al.*, 2005). As compared to other subtypes, the basal-like and HER2 tumors contain the highest rate of mutation. 80% of basal-like tumors with TP53 gene mutation which have been linked to poor outcomes (Cancer Genome Atlas Network, 2012). Currently, all breast cancers patients are tested for the ER, PR, and HER2 proteins expression. ER and PR are assayed by IHC while HER2/neu is examined by FISH. The protein profiling of tumors may provide useful information to design a better therapeutic treatment and predict survival outcomes for breast cancer patients.

1.2.9 Breast cancer diagnosis and treatment

There are different types of treatments available for breast cancer patients. The clinical symptoms observed in breast cancer patients are largely dependent on whether the tumor is confined to the breast or has metastasized. Primary therapies of local breast cancer include surgery (breast-conserving or mastectomy) and radiation therapy. Patients may opt for surgical removal of primary local tumor from the breast (breast-conserving) or to remove part of the breast (mastectomy). These patients may also be subjected to subsequent radiotherapy, chemotherapy, or hormone therapy to eradicate remaining cancer cells residing in the breast (Majure, 2000). Chemotherapy is an alternative treatment options that utilizes drugs to inhibit cell division and eventually the growth of cancer cells. Endocrine therapy involves blocking of hormones in order to attenuate cancer cells from growing (Majure, 2000). Some of endocrine therapies include competitors for ER and inhibitors of estrogen to counteract the effect of estrogen in breast cancer (Normanno *et al.*, 2005). Patients with elevated levels of ER and/or PR (i.e., luminal subtype) are treated with endocrine therapy (Normanno *et al.*, 2005). Other anti-estrogen agents for breast cancer patients include synthetic selective estrogen modulators (SERMs), such as tamoxifen, which acts as ER antagonist in breast cancer tissue, and aromatase inhibitors which inhibit the action of the enzyme aromatase to convert androgens into estrogens (Osborne, 1999). Tamoxifen has an agonist or antagonist effect depending on the molecular profile of its target (Jordan *et al.*, 2001). It reduces the development of breast cancer in both pre- and post-menopausal women who are at risk for the disease (Schlotter *et al.*, 2003). ER status is utilized as an indicator to deduce the sensitivity of breast cancer cells to tamoxifen and aromatase inhibitors (Fabian and Kimler, 2005). Raloxifene, which has anti-estrogenic behavior, is being widely used as a preventive chemotherapy for women who maybe at higher risk of developing of breast cancer (Oseni *et al.*, 2008). Additionally, a new type of endocrine therapy with ER antagonist effect, known as Fulvestrant, has also been developed. Fulvestrant acted by both down-regulating and degradation the ER expression (Kansra *et al.*, 2005). It can also be used after an anti-estrogen

therapy (Tamoxifen®) (Raina, 2004, Nicholson *et al.*, 2007). Targeted therapy is another treatment that utilizes drugs to identify and target specific cancer cells without causing any harm to normal cells. There are two types of targeted therapies available for treatment of breast cancer, which include the use of monoclonal antibodies and tyrosine kinase inhibitors (Crown *et al.*, 2012). One example is the use of PARP inhibitors as targeted therapy against triple-negative breast cancer (Crown *et al.*, 2012). Moreover, HER2/neu is the primary target of humanized monoclonal antibody trastuzumab (Herceptin®), which target the extracellular domain of this receptor and blocks the effects of HER2 in conveying growth signals to breast cancer cells (Kurokawa *et al.*, 2000). Therefore, patients with HER2 amplified (HER2+) tumors are given trastuzumab as monotherapy. Alternatively, this targeted therapy can be supplied to patients in combination with standard chemotherapy (Slamon *et al.*, 2001). However, majority of patients with HER2+ metastatic breast cancer who responded to trastuzumab at initial treatment stage eventually developed resistance within a year of treatment and some patients even relapse despite trastuzumab-based therapy (Cobleigh *et al.*, 1999, Vogel *et al.*, 2002).

Although clinical trials for combinational therapies of anti-estrogen and anti-HER2/neu treatment showed promising results (Kurokawa *et al.*, 2000), it has been reported that HER2-positive breast cancers have lower sensitive to estrogen modulation due to HER2 and ER cross-talk (Kurokawa and Arteaga, 2003). Development of resistance to therapeutic drugs (*de novo* or intrinsic resistance) is still a major barrier to effective treatment of breast cancer. It has been reported that some patients did not respond to therapy due to intrinsic drug resistance and multiple signaling pathways regulating the oncogenesis of breast cancer. Cross-talk between the ER and several growth factor signaling pathways has been widely recognized (Schiff *et al.*, 2004). Even in the absence of estrogen, cancer cells can still use growth factor signaling pathways as a compensatory mechanism and acquire anti-estrogen drug resistance (Schiff *et al.*, 2004).

A new recombinant humanized monoclonal anti-HER2 antibody, Pertuzumab, is currently being investigated. Pertuzumab binds to extracellular dimerization domain of HER2 and inhibits the heterodimerization of HER2 with other HER receptors or EGF receptors, thereby preventing activation of HER2 signaling pathways (Metzger-Filho *et al.*, 2013). Formulation of small molecule tyrosine kinase inhibitor (TKI) may reduce the onset of resistance and can be used in combination with targeted therapies. Example of TKI includes Bevacizumab® which is an anti-angiogenic molecules targeting VEGF-A, and anti-insulin growth factor 1 (IGF-1) therapy (Johnston, 2006). Hence, a multi-targeted therapeutic approach may offer a promising treatment for breast cancer patients.

1.3 Trefoil factors

The human trefoil factors (TFFs) are a family of proteins comprising of three members, which comprise of TFF1 (pS2) that was firstly identified as an estrogen-responsive gene in MCF-7 human breast cancer cell line (Jakowlew *et al.*, 1984, Nunez *et al.*, 1987), TFF2 (Spasmolytic polypeptide, SP) (Tomasetto *et al.*, 1990, Beck *et al.*, 1996), and TFF3 (intestinal trefoil factor, ITF) (Suemori *et al.*, 1991, Schmitt *et al.*, 1996). TFF1 was the first member of the family identified via cDNA cloning of estrogen-responsive gene from a breast cancer cell line (Masiakowski *et al.*, 1982, Jakowlew *et al.*, 1984). TFF2 was almost coincidentally isolated in porcine pancreas (Jørgensen *et al.*, 1982, Thim *et al.*, 1985). Subsequently, TFF3 from rat intestine was the third human trefoil factor described (Suemori *et al.*, 1991). The trefoil proteins have also been identified in other mammals such as rat, mouse and chimpanzee (Thim and May, 2005). TFF peptides are specifically synthesized in mucin producing cells of gastrointestinal (GI) tract and thus are characteristic secretory products of the mucous epithelia. Physiologically, TFF1 and TFF2 are expressed in human stomach mucosa and TFF3 is predominantly expressed in GI tract and colon. Evidence indicated that TFF peptides are involved in maintaining the integrity of GI epithelium by supporting mucosal defense and repair mechanism (Taupin and Podolsky, 2003, Hoffmann,

2004). The gene encoding human TFF peptides are found on chromosome 21q22.3 in a 55 kb gene cluster region (Seib *et al.*, 1997) (Figure 11). TFF peptides have seven conserved cysteines residues, of which six are connected to each other by disulfide bonds in a specific order, forming a three-cyclic structure, whereby the whole peptide chain is folded and shaped like a "clover". This structural motif domain is named as trefoil domain or P-domain (Thim, 1989, Polshakov *et al.*, 1997). Trefoil domain is cysteine-rich module that forms the functional unit for TFF peptides (Wright *et al.*, 1997, Wright, 1998, Thim and May, 2005, Hoffmann, 2006) and confers proteolytic stability and resistance to acidic degradation (Suemori *et al.*, 1991). TFF peptides contain either one or two trefoil domains, which consist of 42 - 43 of highly conserved or semi-conserved amino acids (Thim, 1989, Poulsom *et al.*, 1997). TFF1 and TFF3 each contain one trefoil domain, whereas TFF2 contains two (Figure 11). Both TFF1 and TFF3 have a seventh cysteine residue at the carboxyl terminus of the peptides that facilitates homodimerization and intermolecular interactions with other proteins (Thim and May, 2005). TFF1 and TFF3 can exist in several different molecular forms such as monomers, homodimers and heterodimers. TFF dimers are predicted to be more biologically active than monomers. The monomer and homodimer of TFF1 are more asymmetric than that of TFF3 and such differences may account for the divergent biological activities and specificity (May *et al.*, 2003).

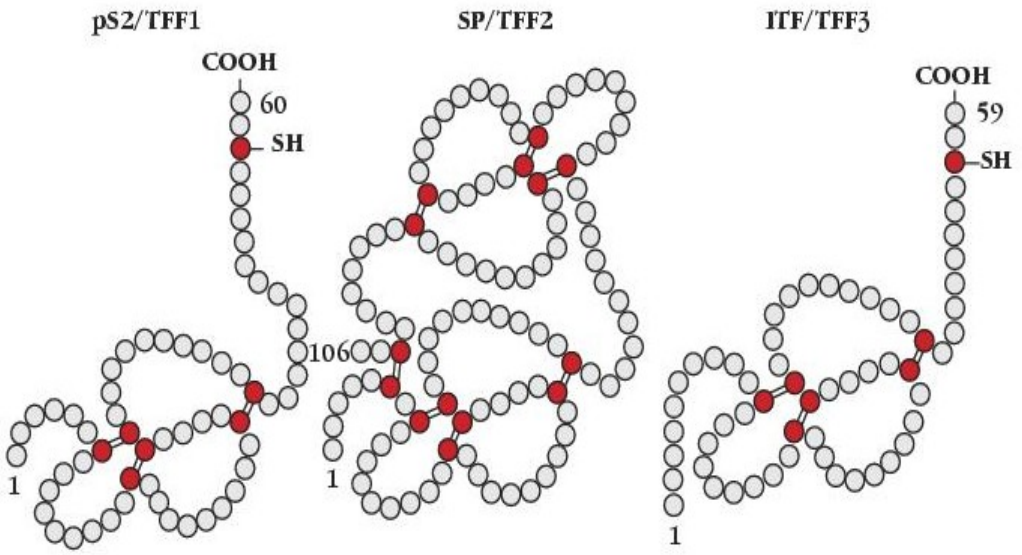


Figure 11: Human TFF peptides. Conserved cysteines residues (red circle) connected with each through disulfide bonds in a specific order to form a three-cyclic structure (Thim and May, 2005). Reproduced with permission.

1.3.1 Gene structure and regulation of TFF

All three human TFF genes are located in clusters on chromosome 21q22.3 in a sequential arrangement within a single 55 kb genomic region in the order telomere-TFF1-TFF2-TFF3-centromere (Hattori *et al.*, 2000) (Figure 12). TFF1 and TFF3 genes contain three exons, while TFF2 contains four exons. These exons encode the amino terminal signal peptide, followed by the trefoil domain, and the carboxyl terminal acidic motif, respectively. Transcription of these three genes is directed towards the centromere. In mouse genome, a 40 kb region located on chromosome 17q is found to resemble the human TFF gene (Ribieras *et al.*, 2001). Conservation of TFF gene cluster between different organisms (mouse and human) implied that TFF expression is partially regulated by a locus activation region (Thim and May, 2005). The promoter region consists of an estradiol responsive element (ERE) (Berry *et al.*, 1989), and also contains a complex enhancer region responsive to the proto-oncoproteins c-Ha-ras, tumor promoter TPA, epidermal growth factor (EGF) and c-jun (Nunez *et al.*, 1989). Several transcriptional factors, regulatory proteins and epigenetic factors including estrogens, progesterone, epidermal growth factor, basic fibroblast growth factor, upstream stimulating

factor (USF), hepatocytes nuclear factor, GATA-6 and Ha-Ras/AP-1 coordinately regulate the cell-type specific expression of TFF genes (Beck *et al.*, 1999, Al-azzeh *et al.*, 2002, Borthwick *et al.*, 2003).

TFF1 promoter region contains an ERE that lies between 332 to 428 bp upstream of the transcriptional start site. Activation of TFF1 expression is induced by estrogen in ER+ mammary carcinoma cells, acting in a synergistic manner with the steroid receptor coactivator-1 (Src-1), via the AP-1 and ERE at the TFF1 promoter (Barkhem *et al.*, 2002a, Barkhem *et al.*, 2002b). Estradiol-mediated regulation of TFF1 gene expression has been observed in mammary carcinoma (Rio *et al.*, 1987), but has not been conclusively demonstrated in other tissues. The presence of other regulatory elements may contribute to the expression of TFF1. An example is GATA-6, which is an endodermal transcription factor expressed in various tissues including pancreas gut, liver, pancreas, and lung. GATA-6 activates expression of TFF1 and TFF2 but not TFF3 (Al-azzeh *et al.*, 2000). Moreover, TFF1 gene is down-regulated by NF-κB and C/EBPβ (Dossinger *et al.*, 2002). In addition to the genetic regulation of TFF1 expression, epigenetic mechanisms also modulate TFF1 expression levels. The proximal promoter/enhancer region of TFF1 gene contains several CpG islands. The TFF1 gene is subject to tissue-specific methylation of its proximal enhancer region and the extent of methylation correlates with its expression (Martin *et al.*, 1997, Fujimoto *et al.*, 2000, Carvalho *et al.*, 2002).

TFF2 was identified as a side product from the purification of insulin from porcine pancreas (Sands and Podolsky, 1996). There is limited knowledge or literature on the transcriptional regulatory elements of this gene but the transcription factors such as GATA-6, STAT-6, upstream stimulating factors (USF) and Peroxisome proliferator-activated receptor gamma (PPARγ) activate TFF2 gene expression (Al-azzeh *et al.*, 2000, Baus-Loncar *et al.*, 2004). In contrast, NF-κB and C/EBPβ down-regulate TFF2 expression (Dossinger *et al.*, 2002).

TFF3 promoter region contains two ERE (Baus-Loncar *et al.*, 2004). Induction of TFF3 expression has been shown to be estrogen-dependent in breast cancer cells (May and Westley, 1997a). The pro-inflammatory cytokine IL-6 induces TFF3 gene expression via STAT3 transcription factor (Tebbutt *et al.*, 2002). TFF3 promoter contains HIF-1 response elements that results in increased TFF3 expression mediated by HIF-1 under hypoxia (Furuta *et al.*, 2001). As demonstrated in TFF1 and TFF2, NF-κB and C/EBPβ down-regulate the expression of TFF3 gene via four NF-κB and two C/EBPβ binding motifs (Dossinger *et al.*, 2002, Loncar *et al.*, 2003).

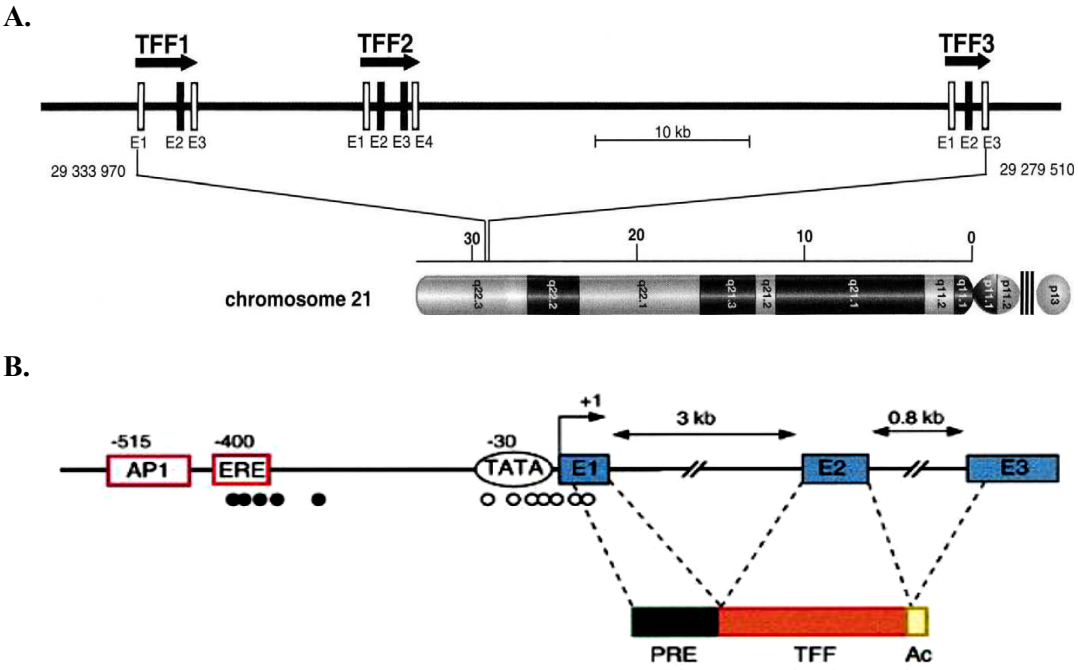


Figure 12: Chromosomal localization of human TFF genes. A, TFF cluster on chromosome 21q22.3. The exons (e.g., E2 or E3) encode human trefoil domains and the first exon (E1) encodes the signal sequences of the precursors of TFF proteins. B. Regulatory elements such as ERE at the promoter region of trefoil factors (Ribieras *et al.*, 1998). Reproduced with permission.

1.3.2 TFF protein

Mammalian orthologous sequence analysis of TFF across different species, including human, rat, mouse, canine, pig, and chimpanzee, shows an overall amino acid sequence conservation of 52% with a 57% homology in the trefoil domain (Thim and May, 2005). The amino acid sequence of the trefoil domain in TFF3 is conserved among mammalian species with an orthology of 67% (Thim and May, 2005). TFF3 shares the relatively low level of amino acid sequence homology of 36% compared to TFF1. However, TFF3 contains many of similar elements that are common to members of TFF family (Sands and Podolsky, 1996). TFF peptides are synthesized with a signal sequence for secretion of 21 or 27 amino acid residues that are proteolytically spliced away when passaging through the endoplasmic reticulum. The secreted mature form of TFF peptides comprises a signal peptide, one or two trefoil domain (s), and a carboxy-terminal acidic domain. Despite having similar isoelectric points, TFF1 and TFF3 differs significantly in their hydrodynamic properties, distribution of surface amino acids and overall charge (Emami *et al.*, 2004).

TFF1 possesses 60 amino acid residues, encoding a 9 kDa protein and a single trefoil domain consists of 42 amino acid residues. TFF2 contains 106 amino acid residues, encoding a 14 kDa protein, and two trefoil domains consisting 43 residues and 42 residues respectively in the first and second trefoil domains. The first trefoil domain in TFF2 differs as it contains an additional amino acid residue. TFF3 consists of 59 amino acid residues, encoding a 7 kDa protein, and contains a single trefoil domain of 42 amino acid residues (Figure 13). In addition to the six conserved cysteine residues in the trefoil motif, TFF1 and TFF3 contain a seventh cysteine residue located three amino acids away from the carboxyl terminus of the protein. This allows homodimerization, via a disulfide bond with the seventh cysteine residue of the other trefoil factors as well as heterodimerization, via intramolecular disulfide bonds with other proteins (May *et al.*, 2003, Thim and May, 2005, Westley *et al.*, 2005). TFF2 has two trefoil domain cysteine residues that can produce an intermolecular disulfide bond (Thim, 1989).

The biological potencies and activities of TFF peptides are dependent on structural differences or interaction with other proteins. Homodimerization is critical for the biological functions of TFF, particularly its ability to stimulate cell migration and proliferation *in vitro* (Marchbank *et al.*, 1998, Calnan *et al.*, 1999, Prest *et al.*, 2002) as well as protection of the gastric against damage *in vivo.* An *in vitro* wound healing model, TFF1 dimer significantly increased cell migration of human colorectal cell line as compared to TFF1 monomer (Marchbank *et al.*, 1998). Dimeric TFF1 significantly reduced gastric damage *in vivo*, while monomeric TFF1 was ineffective. Previous studies utilizing a cell line model of epithelial barrier function have shown that specific mucins interacted with specific trefoil factors to act in a coordinate manner for protection of mucosa (Kindon *et al.*, 1995). Heterodimerization of TFF1 with mucin MUC5AC through C-terminal disulfide interaction promotes mucosal protection in the normal GI tract where these two proteins are co-localized (Longman *et al.*, 2000, Ruchaud-Sparagano *et al.*, 2004). An animal model study suggested that glycosylation of TFF2 is more effective than its non-glycosylated form in the projection against gastric damage (Playford *et al.*, 1995). Homodimerization appears to be required for the protective functions of TFF3 in the GI tract (Carrasco *et al.*, 2004). TFF3 and mucin MUC2 have been shown to co-localize in the GI tract although no direct protein-protein interactions have been elucidated (Longman *et al.*, 2000). Formation of the heterodimer between trefoil factor interactions(z) 1(TFIZ1) and TFF1 is observed in the mucus secretory cells of the stomach. In gastric cancer, the co-expression of these two proteins is deregulated whereby the expression of TFF1 in the absence of TFIZ1 is correlated with both invasive and also metastatic phenotype (May *et al.*, 2009). Piezo1 is a novel TFF1 binding protein which is crucial for TFF1-mediated cell migration (Yang *et al.*, 2014).

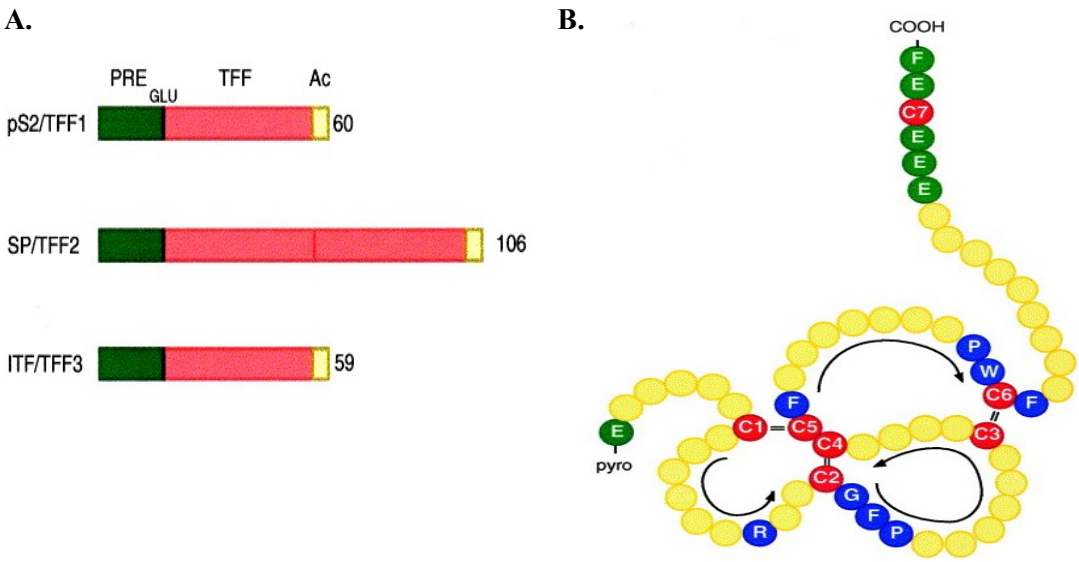


Figure 13: TFF protein. A, Conserved protein primary structure of the three human TFFs, including a signal peptide (PRE), one or two TFF domains (TFF) and a carboxy-terminal acidic domain (Ac). B, Trefoil secondary structure. The conserved cysteine residues in the trefoil domain formed disulfide bond in a C1-C5, C2-C4, and C3-C6 configuration to create three loops reminiscent of a cloverleaf. Conserved cysteine (C7) at the carboxy terminus facilities homo- hetero-dimerazation (Ribieras *et al.*, 1998). Reproduced with permission.

1.3.3 Normal expression patterns and localization of TFF proteins

TFF proteins have protective and restorative functions in normal tissue (GI tract) where they are normally expressed, but they exhibit adverse effect in cancerous tissues where they are pathologically expressed. Normal distribution of these proteins is primarily in the GI tract although they have specific localization within the tract to perform their functions in gastric mucosa.

TFF proteins are usually localized to the gastric mucosa with TFF1 and TFF2 normally expressed in the gastric epithelia. TFF proteins are not only expressed in GI tissues, but are also expressed in other tissues such as bronchial and urogenitary tracts (May and Westley, 1997b, Oertel *et al.*, 2001). TFF1 and TFF3 are expressed in human hypothalamus, gastric juice and urine, suggesting autocrine, paracrine and endocrine functions for these proteins (Rio *et al.*, 1987, Hoffmann and Jagla, 2002). TFF proteins are major components found in the GI mucosa, but each is expressed differentially. TFF1 and TFF2 expression are

found in the gastric epithelial, while TFF3 in the goblet cells of intestine. Both TFF1 and TFF2 are expressed in the stomach but have different expression patterns within this organ. TFF1 is mainly expressed in the superficial cells of the GI tissue (Ruchaud-Sparagano *et al.*, 2004), whereas basal cells of the gastric tissue express TFF2 (Minegishi *et al.*, 2007). TFF1 expression is not observed in the uterus, spleen, muscle, small intestine, lung, kidney, brain, hepatocellular, testis, pancreas, colon, cardiac, and skin (Bonkhoff *et al.*, 1995, Ribieras *et al.*, 1998). Low levels of TFF1 expression are seen in the mammary gland and breast epithelium (Piggott *et al.*, 1991, Hanby *et al.*, 1993). TFF2 is expressed in the Brunner's glands of the duodenum (Ribieras *et al.*, 1998) and TFF3 is predominantly expressed in the small and large intestine, particularly the colon and duodenum (Taupin and Podolsky, 2003). TFF3 has also been shown to be expressed in the lung, salivary glands, the cornea, conjunctiva tissue, brain, and lymphoid tissue (Cook *et al.*, 1999, Jagla *et al.*, 1999, Göke *et al.*, 2001, Emami *et al.*, 2004) where they are postulated to possess function in inflammation and epithelial ulceration.

1.3.4 Expression of TFF3 in human mammary carcinoma

Elevated expression of TFF proteins has been reported in a number of human cancers, including breast, prostate, esophagus, stomach, liver, pancreas and intestines (Emami *et al.*, 2004). Therefore, TFF proteins may have a pivotal function in the development and progression of human cancer.

In the human breast, TFF3 protein is normally present in breast epithelial cells at the lobules and ducts. The function of TFF3 in the human breast may be to protect and maintain the integrity of the epithelial surface (May and Westley, 1997a, Poulsom *et al.*, 1997). Co-expression of TFF1 and TFF3 was observed in breast epithelial cells. Poulsom *et al.* (1997) analyzed the expression of TFF3 in breast cancer tissue by *in situ* hybridization and observed that TFF3 was highly expressed in both non-invasive and invasive lobular and ductal breast cancer. A recent cohort study on breast cancer patients has shown that increased TFF3 expression in 89% of *in situ* mammary carcinoma and in 83% of invasive mammary

carcinoma (Ahmed *et al.*, 2012). In breast cancer cells, TFF3 protein expression was observed to be strongly associated with the expression of estrogen and progesterone receptors. Additionally, it has been shown that the expression of TFF3 is regulated by estrogen (May and Westley, 1997a).

1.3.5 TFF3 signaling

Although TFF3 plays a critical part in tumor progression, the signaling pathways of TFF3 are not fully understood. A receptor for TFF3 protein has not yet been identified to elucidate the mechanism of TFF3 in both normal and pathological contexts. Numerous studies have shown that TFF proteins may indirectly bind to other proteins or secreted factors that may activate signaling cascades for promoting the biological effects of TFF3 protein. Using immunoprecipitation followed by cross-linking, Chinery and Cox (1995) observed that TFF3 binds to a phosphorylated tyrosine-containing peptide of 28 kDa. Another study has demonstrated that TFF3 binds to 50 kDa membrane glycoprotein in the small intestine crypts and also in the mucous cells of the gastric glands (Tan *et al.*, 1997). A recent study speculated that Sp-1 binds to the core region of TFF3 promoter (positioned from -300 bp to -296 bp) resulting in increased TFF3 promoter activity and the subsequent stimulation of TFF3 expression (Sun *et al.*, 2014). Additionally, ERG is a ETS transcription factor and directly binds to ETS response elements in the TFF3 promoter in ERG-rearranged prostate cancer cell lines to induce invasive potential of castration-resistant prostate cancer (Rickman *et al.*, 2010). Signaling pathways that have been identified for potentiating the biological functions of the TFF3 protein include EGF, G-protein, and cyclooxygenase (COX).

(i) Epidermal Growth Factor (EGF)

Although TFF3 has not been reported to bind directly to a receptor, it is able to signal through specific pathways via indirect activation. Previous studies have proposed that TFF3 transmits signal indirectly through the activation of EGF-R (Liu *et al.*, 1997). EGF-R signaling pathway is important for tumor growth and over-expression of EGF-R is correlated

with metastasis potential, poor survival and higher relapse rates in human tumors (Radinsky *et al.*, 1995, Ozgul *et al.*, 1997). Upon stimulation by TFF3, EGF-R is activated and receptor tyrosine protein kinase activity is enhanced. Treatment of gastric cancer cell lines with TFF3 resulted in phosphorylation of the EGF-R, indicating that TFF3 has the ability to indirectly activate EGF receptor (Liu *et al.*, 1997, Kanai *et al.*, 1998, Taupin *et al.*, 1999, Kinoshita *et al.*, 2000b). In addition, the pro-invasive effects of TFF1 and TFF2 were abrogated by treatment with EGF-R tyrosine kinase inhibitor and/or with the use of a dominant negative form of the EGF-R (lacking intracellular domain), suggesting that this pathway is essential for the pro-invasive function of these proteins. However, the pro-invasive effect of TFF3 was not affected by EGF-R tyrosine kinase inhibitors under the same experimental conditions, indicating that TFF3 induced invasion of kidney and colon cancer cells independent of EGF-R (Rodrigues *et al.*, 2003a). This raises the possibility that TFF3 may exert its functions through both receptors and signaling pathways (Rodrigues *et al.*, 2003a). This observation is supported by Yong *et al.* (2013) have identified a TFF3 specific binding receptor in the intestinal epithelial cells. The authors believed that EGF-R is unlikely to be the TFF3 receptor (Yong *et al.*, 2013). Several studies have demonstrated that TFF3 may transmit extracellular signals and promote cell proliferation and migration through activation of EGF-R (Hoffmann, 2007, Li *et al.*, 2011). Furthermore, the pro-angiogenic functions of TFF1 appear to be regulated in an EGF-dependent manner as pharmacological inhibition of EGF-R tyrosine kinase activity resulted in decreased TFF1-induced angiogenesis (Rodrigues *et al.*, 2003b).

(ii) Heterotrimeric G-proteins

Another potential signaling cascade activated by the TFF peptides is the heterotrimeric G-protein pathway. There is no experimental data to show that TFF peptides directly bind to G-protein receptors. Several studies indicate that the G-protein signaling pathway is important for the mitogenic functions of TFF1 and TFF3 (Faivre *et al.*, 2001, Rodrigues *et al.*, 2001). The TFF3- and TFF1-induced invasion of kidney and colonic epithelial cells were abrogated by the pharmacological inhibition of critical elements of the G-

protein signaling machinery including Rho small GTPase and thromboxane A2 receptor (TXA2-R). In contrast, the constitutively activated forms of heterotrimeric G-proteins Gαq (AGαq), Gα12, Gα13 (AGα12/13) and TXA2-R mimetic led to increased invasion of kidney and colonic epithelial cells promoted by these two proteins (Rodrigues *et al.*, 2001). Additionally, over-expression of TFF1 resulted in increased invasion mediated by Rho-independent and TXA2-R-dependent pathways (Rodrigues *et al.*, 2001) and ablation of Gβγ heterodimers resulted in decreased TFF-induced cellular invasion (Faivre *et al.*, 2001).

(iii) Cyclooxygenases COX-1 and COX-2

The pro-inflammatory molecules COX-1 and COX-2 have been identified as potential signaling mediators of the TFF peptides. Both molecules are proposed to contribute in the pro-invasive and pro-angiogenic functions of TFF peptides although experimental evidence is very limited. Expression of COX-2 has been postulated in tumor initiation and progression by regulating cell proliferation, migration, and invasion (Young *et al.*, 1992). Its oncogenic effect can be induced by several growth factors and oncogenes such as EGF-R tyrosine kinase, and pro-inflammatory cytokines (Vane *et al.*, 1998). It has been shown that inhibition of COX-1 and COX-2 led to the ablation of TFF1-induced invasion and angiogenesis (Rodrigues *et al.*, 2001, Rodrigues *et al.*, 2003a, Rodrigues *et al.*, 2003b). Therefore, TFF1 induces COX-2 expression to promote cellular invasion in human colonic cells (Emami *et al.*, 2001). TFF3 binds to intestinal mucosa and enhances production of nitric oxide via activation of cyclooxygenases (COX-2) pathway (Rodrigues *et al.*, 2001).

1.3.6 Biological functions of TFF3

TFF peptides have been implicated in stabilization of mucus and stimulation of normal epithelial restitution during mucosal repair. TFF peptides are rapidly expressed and secreted in an autocrine manner during GI injury. The secreted TFF peptides function as motogens to assist migration of epithelial cells into the wound, thereby providing a physical barrier in a process referred to restitution (Taupin and Podolsky, 2003) (Figure 14).

Additionally, TFF proteins are potent inhibitors that resist apoptosis and anoikis during the process of migration of the cell. Therefore, the biological functions of TFF proteins include (i) forming and stabilizing the mucus barrier, (ii) promoting rapid mucosal repair (restitution), (iii) modulating mucosal differentiation as well as (iv) modulating mucosal immune response (Hoffmann, 2004).

First lines of evidence suggesting that TFF proteins play a protective role arise from animal studies whereby rats were treated with the prostaglandin inhibitor indomethacin that causes ulceration of the GI tract. Combined prophylactic treatment of these animals with epidermal growth factor (EGF) and TFF3 led to remarkable reduction of gastric damage (Chinery and Playford, 1995, Playford *et al.*, 1995). Subsequently, this observation was confirmed by *in vivo* studies which showed administration of TFF2 and TFF3 proteins to confer protective and beneficial effects against chemical-induced damage of the gastric mucosal at a level comparable to prostaglandin-mediated cytoprotection (McKenzie *et al.*, 2000, Babyatsky *et al.*, 2009). Furthermore, knockout mice lacking TFF3 expression were more susceptible to dextran sodium sulfate-induced colitis and had a higher mortality rate than wild-type mice (Mashimo *et al.*, 1996). Mice with TFF1 and TFF3 deficiency exhibit impaired mucosal healing and eventually die due to high rate of apoptotic events of colon cells associated with severe colitis (Lefebvre *et al.*, 1996, Mashimo *et al.*, 1996). *In vitro* model systems also demonstrated that increased expression of TFF2 and TFF3 proteins significantly reduced colonic damage induced by multiple toxins insults on colon cancer cells and the protective effects of the TFF peptides were enhanced with co-expression of mucin glycoproteins (Kindon *et al.*, 1995). The function of TFF2 and TFF3 are mediated through the epithelial (E)-cadherin/catenin complexes, which is one of the common motogenic targets during cell migration (Liu *et al.*, 1997). TFF3 promoted cell migration by inhibition of E-cadherin function and tyrosine phosphorylation of β-catenin (Liu *et al.*, 1997, Efstathiou *et al.*, 1999). TFF3 also can stimulate down-regulation of E-cadherin, α- and β-catenin that inducing subsequent decrease in cell adhesion for enhancing cellular invasion and migration of cancer

cells (Liu *et al.*, 1997). The prophylactic effects of TFF proteins have been demonstrated in both animal and cell line models of GI tract injury. These data support that TFF proteins serve as beneficial factors in the maintenance of the epithelium of GI tract. Several mechanisms have been shown would be contributed to the protective and tumor suppressive roles of TFF proteins. For example, transgenic mice with over-expression of growth hormone (GH) exhibit elevated expression of TFF3 in the colon with enhanced survival, and mucosal repair during recovery from experimental colitis. These observations suggested possible interaction or cross-talk between EGF-R signaling with TFF3 expression in mucosal repair mechanism (Williams *et al.*, 2001). Furthermore, the motogenic function of TFF3 peptides in restitution of GI epithelium is probably through a TGFβ-independent pathway (Dignass *et al.*, 1994, Sands and Podolsky, 1996).

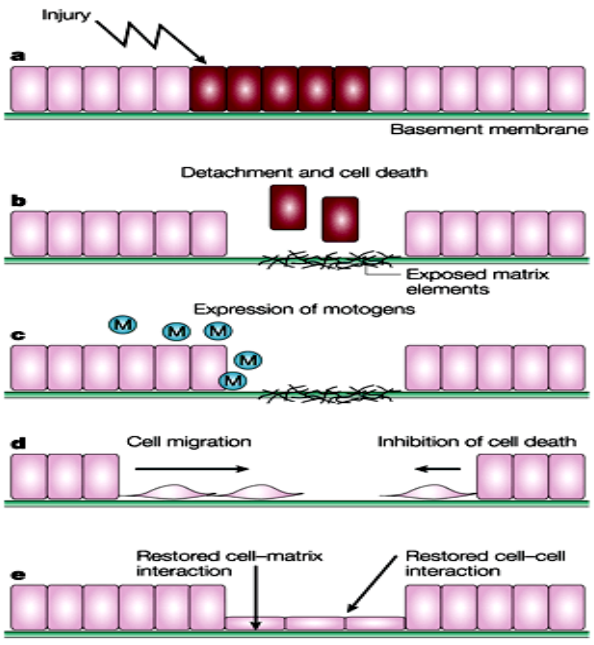


Figure 14: The different steps of mucosal restitution. a. Local mucosal damage. b, cellular detachment and apoptosis immediately occurred. c, activation of adjacent mucosal cells at the wounded areas to release motogenic agents (motogens; M) such as trefoil factors and to initiate the mechanism of restitution or repair. d, this process followed by migration of epithelial cells and inhibition of cell death. d, which is accompanied by mechanisms that prevent detachment-induced cell death. e, restoration of cell-matrix interaction and cell-cell interaction at the wounded areas (Taupin and Podolsky, 2003). Reproduced with permission.

1.3.7 Oncogenic potential of TFF3

TFF3 may serve as either tumor suppressor genes or potential tumor promoting factors depending on tissue localization. Although TFF3 protein play a protective and restorative role in the GI tract where it is normally localized, TFF3 protein is over-expressed in various tumor tissues including those of pancreatic, prostate, breast, liver, endometrium, ovary, uterus, esophagus, and bladder (May and Westley, 1997b, Emami *et al.*, 2004). Many reasons can account for this duality in function including the positive association between inflammation and increased TFF3 expression in the microenvironment of the tumor, epigenetic modification of TFF3 expression, or activation of oncogenes that increased the expression of TFF3. However, the mechanisms of TFF3-mediated activation of oncogenes in cancerous cells have yet to be elucidated. Increasing experimental studies demonstrated the tumor promoting actions of TFF peptides implicated in tumor growth and progression.

(i) Proliferation

There is very few evidence demonstrating the role of TFF peptide in regulating cell cycle progression. Autocrine production of TFF3 significantly enhanced proliferative capacity in mammary carcinoma cells (Xu *et al.*, 2005, Kannan *et al.*, 2010). Forced expression of TFF3 in mammary carcinoma cell lines markedly increased the total cell number as a result of enhanced cell proliferation and survival (Kannan *et al.*, 2010). However, over-expression of TFF3 suppressed growth of human colon cancer cells and was shown to function as an inhibitory factor for proliferation of colonic neoplasm (Uchino *et al.*, 2000). Furthermore, depleted expression of TFF3 using an antisense oligonucleotide significantly reduced proliferation of human gastric carcinoma cell line (Chan *et al.*, 2005a).

(ii) Apoptosis

Resisting apoptosis is one crucial characteristic during oncogenesis. Studies have shown that all the TFF family members play an anti-apoptotic role. TFF1 has been demonstrated to prevent gastric carcinoma cells from undergoing anoikis, chemical-induced apoptosis, as well as BCL-2-antagonist of cell death (BAD)-induced apoptosis (Bossenmeyer-

Pourie *et al.*, 2002). Forced expression of TFF3 in colon carcinoma cells prevented the cells against serum starvation-induced and drug-induced apoptosis, which is dependent on PI3K activity (Kinoshita *et al.*, 2000a, Taupin *et al.*, 2000a). Autocrine TFF3 stimulated survival of mammary carcinoma cells increased BCL-2 protein expression (Kannan *et al.*, 2010). A study reported that TFF3 activated Akt/protein kinase B survival pathway in the process of mucosal repair (Taupin *et al.*, 2000a). The p53-dependent apoptosis in gastric carcinoma cell lines following treatment with etoposide was markedly abrogated by forced expression of TFF3 (Taupin *et al.*, 2000a). Concordantly, antisense TFF3 significantly increased adriamycin-induced apoptosis in gastric carcinoma cells (Chan *et al.*, 2005a).

(iii) Anoikis

Anoikis is a form of anchorage-dependent cell death program induced by cell detachment. It is a relatively important event for oncogenic transformation as well as cancer progression. It has been observed that forced expression of TFF3 prevented anoikis in intestinal epithelial cells, which is mediated by a survival signaling pathway linked to NF-κB, a major mediator of TFF-promoted cell survival. Besides that, the stable expression of a mutant form of the NF-κB inhibitor (I-κB) in cells resulted in a significant reduction against anoikis effect of TFF3 (Chen *et al.*, 2000). A study confirmed that TFF3 protected intestinal epithelial cells from anoikis (Regalo *et al.*, 2005) and induced oncogenic transformation of an normal immortalized human mammary epithelial cell line (Xu *et al.*, 2005). TFF3 over-expression in mammary carcinoma cells prevented tamoxifen-induced anoikis (Kannan *et al.*, 2010). Depleted expression of TFF3 inhibited anchorage-independent growth of human mammary carcinoma cells. Additionally, over-expression of TFF3 greatly enhanced colony formation in tamoxifen-resistant mammary carcinoma cells, while depleted expression of TFF3 restored sensitivity of tamoxifen-resistant cells to the same drug (Kannan *et al.*, 2010). Additionally, knockdown of TFF3 significantly inhibited colony formation of gastric carcinoma cells in soft agar (Chan *et al.*, 2005a) and suggesting that TFF3 prevents anokis.

(iv) Migration and invasion

One of the protective functions of the TFF peptides is to serve as motogens to assist in the restitution of damaged mucosal or epithelial tissue but this function may be co-opted in tumor metastasis to promote tumor cell migration and invasion. TFF3 and TFF1 are motogenic in both normal and cancer cells. TFF3 greatly promoted and enhanced migratory and invasive capacities of human colon carcinoma cells (Rivat *et al.*, 2005). Numerous studies have indicated over-expression of TFF3 to be involved in disrupting cell morphology and polarity in colon carcinoma cells by modulating the function of E-cadherin/catenin complex, which typically maintains normal cell to cell contacts (Efstathiou *et al.*, 1998, Meyer zum Buschenfelde *et al.*, 2004). Disruption of these cell to cell contacts and cell polarity resulted in EMT associated with the stimulation of cellular invasion and migration in the metastasis of colon carcinoma cells (Meyer zum Buschenfelde *et al.*, 2004). TFF3 has been shown to promote migration and invasion by rat fibroblast cells by increased the expression of metalloproteinase-9 (MMP-9) and β-catenin as well as simultaneously decreased the expression of E-cadherin and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Chan *et al.*, 2005b). Furthermore, the TFF3-mediated migration of normal and oncogenically transformed bronchial epithelial cells was demonstrated to be p44/42 MAPK-dependent (Graness *et al.*, 2002).

(v) Angiogenesis

Besides the functional role of TFF3 in resisting apoptosis and promoting cell invasion and migration, another oncogenic effect of TFF3 is the ability to promote angiogenesis, which is necessary for tumor growth and dissemination (Andre *et al.*, 2000). Physiological angiogenesis is fundamental in normal tissue homeostasis but this process is co-opted in tumorigenesis to promote survival and the growth of tumor. *In vitro* and *in vivo* studies have demonstrated that TFF peptides may serve as pro-angiogenic factors (Rodrigues *et al.*, 2003b, Emami *et al.*, 2004). Exogenous recombinant TFF protein (rhTFF1) promoted HUVEC tubule formation and the angiogenic responses of TFF1 similar to that observed with VEGF-A and

transforming growth factor-alpha. It has been shown that TFF1 was connected to the activation of VEGF receptor, cyclooxygenase (COX-2), and EGF-R pathways (Rodrigues *et al.*, 2003b). The expression of COX-2 greatly reduced apoptotic activity, increased tumor *de novo* angiogenesis, and promoted invasion and metastasis (Tsujii *et al.*, 1998). Nitric oxide (NO) is a growth factor for inflammation as well as tumor vascular formation (Tan *et al.*, 1999). Exogenous TFF3 has been reported to stimulate the formation of NO by inducing nitric oxide synthase for blood vessel formation (Rodrigues *et al.*, 2001). Concordantly, IHC analysis of gastric carcinoma tissues (Dhar *et al.*, 2005) and breast cancer tissue (Ahmed *et al.*, 2012) demonstrated that increased expression of TFF3 is significantly associated with enhanced tumor vascularity (Dhar *et al.*, 2005, Ahmed *et al.*, 2012).

1.3.8 Clinical correlation of TFF3 in mammary carcinoma

Mounting evidence has recently indicated a clinical association between the expression of TFF3 and the prognosis of breast cancer patient. It has been shown that increased expression of TFF1 and TFF3 mRNA were observed in lobular carcinoma *in situ*, invasive lobular carcinoma and invasive ductal carcinoma (Emami *et al.*, 2004). TFF3 mRNA has also been identified as a prognostic biomarker for breast cancers negative for both estrogen and progesterone receptor (Doane *et al.*, 2006). Kaplan-Meier analysis of breast cancer patients (ER+) treated with tamoxifen has demonstrated that elevated TFF3 mRNA expression is significantly associated with worse disease-free survival (Miller *et al.*, 2005, Kannan *et al.*, 2010). Concordantly, Kannan *et al.* (2010) observed a positive correlation between increased TFF3 mRNA expression and poor survival outcome not only in breast cancer patients but also patients treated with tamoxifen (Kannan *et al.*, 2010). IHC analysis of breast cancer tissues indicated that a negative association between TFF3 protein expression and labeling of cell proliferative biomarker Ki-67 (Ahmed *et al.*, 2012).

The pro-angiogenic function of TFF peptides has been demonstrated using recombinant TFF peptides to induce formation of tube-like structures by HUVEC in the

Matrigel matrix substratum, and capillary vessel formation in CAM assay (Rodrigues *et al.*, 2003b). Thus, the angiogenic activity of TFF peptides is comparable to that stimulated by VEGF and transforming growth factor-alpha. Furthermore, the association between the expression of TFF3 protein and microvessel density (measured by CD34 biomarker staining) has previously been reported in gastric carcinoma tissue (Dhar *et al.*, 2005). A recent cohort study on breast cancer patients, in which endothelial marker CD31 or CD34 expression were examined, has also shown a strong positive correlation between TFF3 protein expression and microvessel density, (Ahmed *et al.*, 2012).

TFF3 promotes invasive and metastatic phenotype in mammary carcinoma cells leading to tumor progression. During metastasis, TFF3 increases the penetration of neural sheath and the intravasation of lymphovasculature. The role of TFF3 protein expression in metastasis of breast cancer has not been extensively studied, but TFF1 and TFF3 mRNA had been identified as a marker for predicting micrometastatic breast cancer in a microarray analysis of early breast cancer patients (Mikhitarian *et al.*, 2005). Another array-based study has shown TFF1 and TFF3 to be two genes most highly and significantly associated with breast cancer metastatic to bone (Smid *et al.*, 2006). Concordantly, increased expression of TFF3 in mammary carcinoma cells was associated with invasive phenotype and metastatic potential, suggesting that continued involvement of TFF3 in the process of metastasis to facilitate the colonization of cancer cells in bone (Ahmed *et al.*, 2012). Additionally, the expression profiling of breast cancer was analyzed using serial analysis of gene expression (SAGE), indicating TFF1 and TFF3 to be signature targets that are expressed in mammary carcinoma and absent in the circulation and bone marrow (Bosma *et al.*, 2002). Therefore, TFF1 and TFF3 have been used as biomarkers for detecting disseminated breast cancer (Lacroix, 2006).

1.4 Angiogenesis

Angiogenesis is an essential process where new blood vessels are formed or new capillaries are grown from pre-existing vasculature (Folkman, 1971). This phenomenon is a tightly controlled and regulated event, which occurs in several normal physiological processes such as reproduction, growth and development, and wound repair (Folkman, 1971). However, angiogenesis can switch to a pathogenic process during tumor growth and metastasis as well as in other diseases such as atherosclerosis and endometriosis (Banerjee *et al.*, 2007).

The angiogenic process is pivotal in tumor growth whereby tumor progression highly dependent on blood vessel recruitment and tumors response to secretory angiogenic factors (Folkman *et al.*, 1971). Therefore, inhibition of tumor angiogenesis can be a potential therapeutic strategy to regress tumor growth (Folkman, 1971). In the past, many research studies related to tumor angiogenesis have been performed to prove that inhibition of angiogenesis could attenuate tumor growth. Recently, preclinical studies of angiogenesis inhibitors have shown that anti-angiogenic therapies may have great potential in the treatment of cancers (Tabruyn and Griffioen, 2007). The Food and Drug Administration (FDA) has approved several therapeutics strategies for the treatment of angiogenesis-dependent diseases including Avastin for colorectal cancer and Tarceva for lung cancer (Tabruyn and Griffioen, 2007).

1.4.1 Mechanism of angiogenesis

Both normal and cancer cells require adequate oxygen and nutrient supply, and the removal of waste products from metabolic processes and sustaining survival. Normally, cells and tissues depend on physiological vasculogenesis referred to as *de novo* angiogenesis to generate vasculature that can meet their metabolic demands (Papetti and Herman, 2002). Cancer cells initially supply their own oxygen and nutrients from the pre-existing vasculature by mimicking normal angiogenesis. Consequently, tumors remain dormant and still able to

survive through the pre-existing vasculature and may not need to induce tumor angiogenesis (Holash *et al.*, 1999).

Tumor angiogenesis is a pathological event resulting from the use of normal angiogenic mechanism, where vasculature is remodeled by new capillaries formation from pre-existing vessels (Papetti and Herman, 2002). Normal angiogenesis provides an adequate source of oxygen and nutrients to the developing or healing tissues. Within a tumor, there is limited amount of nutrients available for actively dividing cells (Folkman, 1971, Jain, 1987). Consequently, tumor cells induce development of new vessels from pre-established vasculature, thereby producing a vascular network that delivers oxygen and nutrients for survival and propagation of tumor cells.

In the physiological angiogenesis, new vessels develop regularly from normal vasculature comprised of mature vessels and maintained by the combination of pro- and anti-angiogenic molecules (Jain, 2001). Endothelial cells (EC) are among the more quiescent and genetically stable cells in the body. Unlike normal blood vessels, tumor vessels generated in pathological angiogenesis are structurally and functionally abnormal. Characteristics of tumor angiogenesis include the presence of highly proliferative EC and atypical morphology of the intratumoral vasculature (Rajotte *et al.*, 1998). The hierarchy of capillaries, arterioles, and venules which is characteristics of normal vasculature is distorted in tumors (Baish and Jain, 2000). EC, pericytes and smooth muscle cells, and the basement membrane of tumor blood vessels are poorly organized (Baluk *et al.*, 2005). Under normal circumstances, EC do not associate with normal blood vessel. However, vasculature produced during tumor angiogenesis becomes hyperpermeable and poorly organized that generally results in high interstitial pressure in most tumors (Baluk *et al.*, 2005). In addition, pericytes are not tightly attached to EC and the vessel wall may weaken and increase the risk of metastasis of cancer cells (Baish and Jain, 2000).

The angiogenic event is highly toned by combination of pro-angiogenic and anti-angiogenic factors (Huang and Bao, 2004, Adams and Alitalo, 2007). An imbalance of either

pro- and anti-angiogenic factors will thus trigger the tumor angiogenesis cascade. When the angiogenic state is favorable, new blood vessel is formed with activation of EC and the removal of mural cells (pericytes) from pre-established vasculature (Figure 15). The absence of these pericytes initiates MMPs to proteolytically degrade the basement membrane of the vasculature (VBM) and ECM (Moses, 1997). The growth of endothelial sprouts (also known as angiogenic sprouting) from pre-existing blood vessel requires a change of cell polarity, induction of motility and invasive capacity, the decrease of cell-cell contact and the degradation of the ECM. Angiogenic sprouting is then followed by an outgrowth of EC, which is regulated by a gradient of pro-angiogenic factors such as VEGF-A. These pro-angiogenic factors are released or produced by VBM and a variety of cell types including tumor cells, fibroblasts, and immune cells. The EC continue to proliferate until they start to form microvessel. After formation of the small blood vessel, recruitment of mesenchymal cells to the vessels occurs, where the cells subsequently differentiated into pericytes and the cell to cell connections between pericytes and EC is substantially established. Finally, the development of stable and mature blood vessels terminates the process. The cells differentiate in a structure identical to capillaries in order to create a vascular network and blood flow that is crucial for cancer development (Adams and Alitalo, 2007).

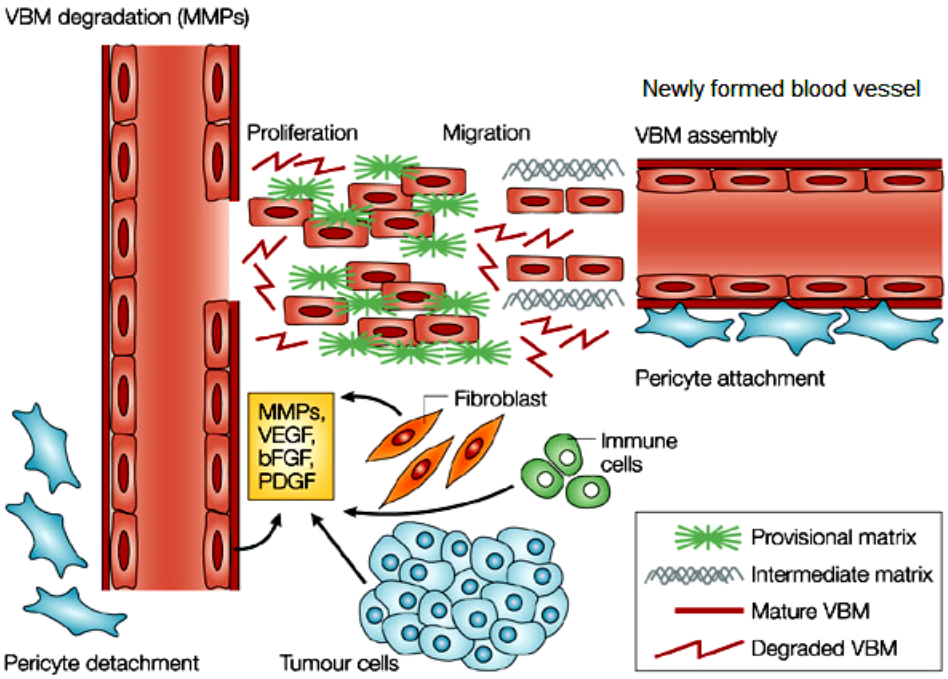


Figure 15: Tumor angiogenesis cascade. Tumor angiogenesis involved degradation and reformation of the vascular basement membrane (VBM). Basement membrane, fibroblasts and immune cells, and tumor cells release a variety of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF). Pro-angiogenic factors and matrix metalloproteinases (MMPs) stimulate structural changes and degradation of basement membrane. These angiogenic factors also induce recruitment of pericytes to stabilize the newly formed blood vessels (Nussenbaum and Herman, 2010).

1.4.2 Angiogenesis and tumor progression

Angiogenesis may influence the ability of tumor outgrowth and metastasis (Folkman *et al.*, 1971). Folkman *et al.* (1971) first discovered the effect of angiogenic factors in tumor progression and also reported the induction of angiogenesis by angiogenic factors secreted from tumor cells. Tumor cells require diffusion of nutrient supply from the interstitial space in the absence of angiogenic factors where tumor usually do not grow beyond 3 - 4 mm in diameter (Folkman, 1990). Laboratory experiments have been done to prove that tumors release angiogenic factors by transplantation of melanoma (Greenblatt and Shubi, 1968) or choriocarcinoma cells (Ehrmann and Knoth, 1968) and that blood vessel proliferation was promoted even in the presence of a filter barrier between host and tumor. These findings support that tumor angiogenesis is promoted by angiogenic factors that are synthesized by

tumor cells. In the absence of vascular support, tumors are restricted with an inadequate supply of oxygen and nutrient and undergo necrosis or apoptosis. Consequently, tumor growth is reduced or remains dormant (Holmgren *et al.*, 1995, Parangi *et al.*, 1996). To overcome this condition, tumor cells induce formation of capillaries by splitting from the original vessel (Carmeliet and Jain, 2000). Tumor angiogenesis significantly contributes to tumor progression by supplying nutrients and oxygen crucial for cancer growth as well as providing a route for dissemination of tumor cells through the bloodstream to secondary organs and form metastases (Cao, 2005).

The process of "angiogenic switch" describes the process of transition from a pre-vascular hyperplasia to a highly vascularized tumor. It is highly regulated by the fine-tuned equilibrium between pro- and anti-angiogenic factors released from tumor cells and/or stromal cells in tumor interstitium (Bergers and Benjamin, 2003). The production of pro-angiogenic factors are also influenced by external factors such as glucose deprivation, cellular acidosis, hypoxia or by deregulation of oncogenes or tumor suppressor genes (Arbiser *et al.*, 1997, Pugh and Ratcliffe, 2003, North *et al.*, 2005). The equilibrium between pro- and anti-angiogenic function prevents tumor outgrowth, which is known as tumor dormancy (Gelao *et al.*, 2013). The shift in equilibrium towards pro-angiogenic functions is associated with tumor and subsequently dissemination of tumors (Gelao *et al.*, 2013) (Figure 16).

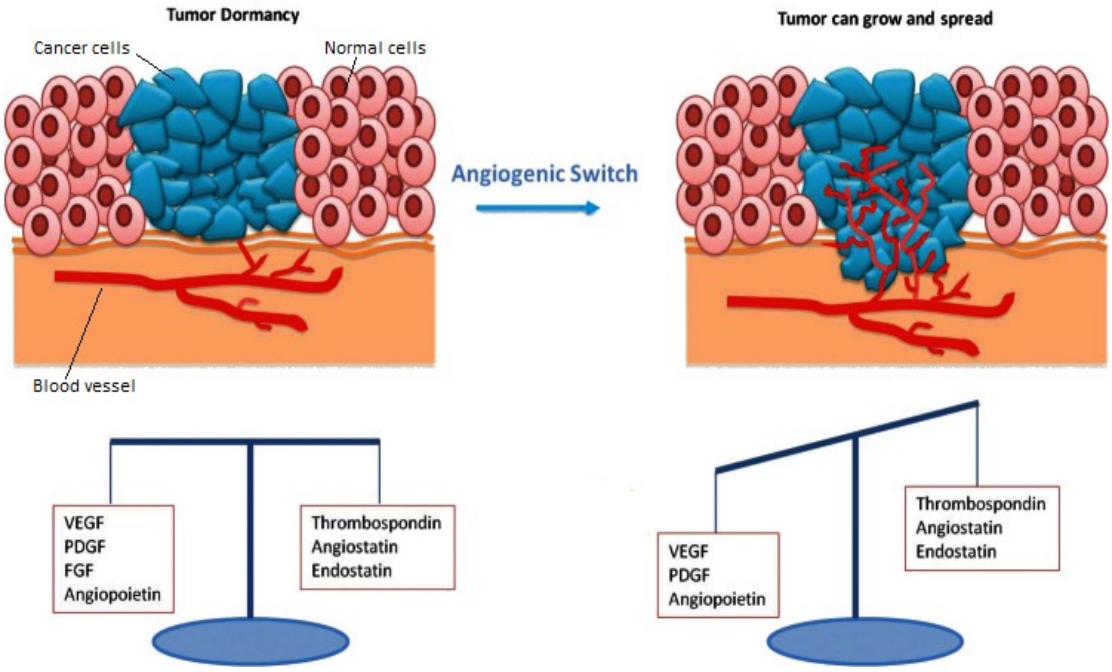


Figure 16: Angiogenic factors in tumor dormancy and growth. An equilibrium between pro-angiogenic and anti-angiogenic factors is found in the tumor dormancy. When this equilibrium is distorted by the prevalence of pro-angiogenic factors, tumor can grow and spread (Gelao *et al.*, 2013).

The angiogenic switch can take place at different stages during tumor progression based on the specific characteristics of the tumor as well as the tumor interstitium (Figure 17). Majority of tumors begin growing as avascular nodules. The angiogenic switch that favors tumor angiogenesis is required to ensure exponential tumor growth. Tumor angiogenesis process involves perivascular detachment, recruitment of EC, activation of EC by angiogenic factors, angiogenic sprouting, stabilization new vessels by perivascular and finally vasculature formation (Cameron *et al.*, 2005). This blood vessel formation allows nutrients to be delivered to hypoxic cells and to the necrotic areas of the tumor for tumor growth and expansion (Bergers and Benjamin, 2003). Therefore, the effect of angiogenic factors secreted by tumor and the regulatory molecules in ECM as well as proteolytic activities in ECM remodeling within the tumor interstitium may contribute to tumor angiogenesis for disseminating cancer cells to distant organs (Mott *et al.* 2004; Sottile 2004).

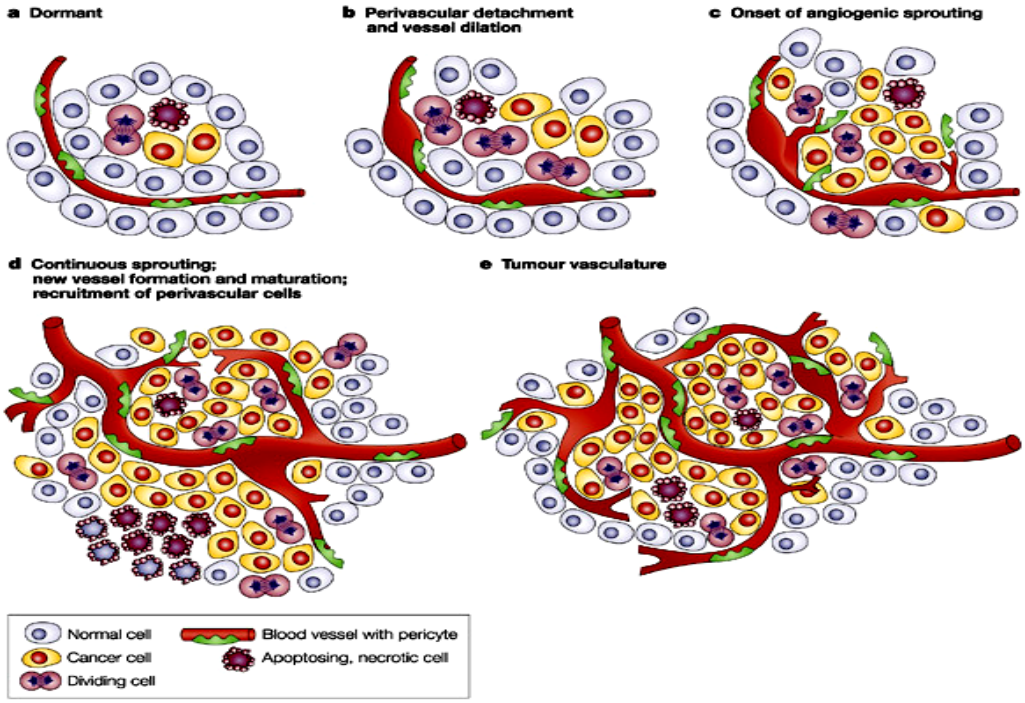


Figure 17: Angiogenic switch and the process of pathological angiogenesis in tumor progression. The angiogenic switch is a distinct step in tumor development that can occur at different stages in the process of tumor progression. a, tumor begins as an avascular masses (dormant). b, the 'angiogenic switch' has to occur to promote tumor growth. The switch started with perivascular detachment and vessel dilation. c, angiogenic sprouting. d, new vessel formation and maturation. e, formation of vasculature in tumor to supply oxygen and nutrient to the cancer cells (Bergers and Benjamin, 2003). Reproduced with permission.

1.4.3 Regulators of angiogenesis

Regulators of angiogenesis are secreted from various cell types such as immune cells, tumor cells and stromal cells or (Folkman, 2003). In normal tissues, anti-angiogenic factors are in excess and maintain the tissue vascularization in a quiescent state. Tumor angiogenesis results from the up-regulation of pro-angiogenic factors and the down-regulation of normal expression level of anti-angiogenic factors (Hanahan *et al.* 1996; Sund *et al.* 2005).

Tumor cells produce many angiogenic factors to induce endothelial cells proliferation and differentiation. Example of angiogenic factors includes interleukin-8 (IL-8), VEGF-A, platelet-derived growth factor (PDGF), angiopoiteins (Ang), acidic and basic fibroblast growth factor (aFGF and bFGF) (Folkman 2003). Among these, IL-8 and VEGF-A are the most pivotal regulators of angiogenesis (Ferrara *et al.*, 2003). FGF and PDGF induce EC

proliferation and migration by interaction with cognate receptors in the EC (Battegay *et al.*, 1994, Friesel and Maciag, 1995). Ang promotes sprouting of vascular remodeling and angiogenesis. Ang-1 promotes the maturation of blood vessel and thus inhibits angiogenesis. Ang-2 antagonizes Ang-1 activity and allows the growing blood vessel to be more responsive to additional angiogenic factors stimulation (Augustin *et al.*, 2009).

Angiogenesis is inhibited by anti-angiogenic factors such as thrombospondins (Tsp), endostatin, angiostatin, interferon-α and -β (INF-α and -β) (Huang and Bao, 2004). TSP-1 is a large ECM glycoprotein and proteolytic fragments (Good *et al.*, 1990). TSP-1 directly inhibits angiogenesis by decreasing EC migration and survival or through indirect action of TSP-1 molecules on activation of transforming growth factor (TGF) and matrix metalloproteinases (MMPs) to inhibit tumor growth (Lawler, 2002, Huang and Bao, 2004). Endostatin is produced by cleavage of collagen XVIII (O'Reilly *et al.*, 1994). Endostatin stabilizes newly formed endothelial tubes in the regions of the tumor where new blood vessel formation is occurring that specifically inhibits endothelial proliferation and tumor growth (Li *et al.*, 2003b). Finally, angiostatin is a fragment of plasminogen and has been demonstrated to be an inhibitor of both neovascularization and additionally metastatic dissemination (O'Reilly *et al.*, 1994). Thereby, the genetic and additional epigenetic events within the tumor interact with the cocktail of pro- and anti-angiogenic factors to induce an angiogenic phenotype in tumor cells (Hanahan *et al.* 1996) and thus initiate tumor angiogenesis.

1.4.4 Vascular Endothelial Growth Factor A (VEGF-A)

VEGF-A is an angiogenic-promoting protein that has a pivotal role in regulating physiological and pathological angiogenesis. It is a member of the VEGF family comprise of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. The members possess a VEGF homology domain and bind to their tyrosine kinase receptors, VEGFR-1, -2, and -3 (Ferrara *et al.*, 2003). VEGF-A is known as a tumor-produced cytokine and is implicated in normal tissue and tumor-associated angiogenesis. It promotes

proliferation and tube formation in endothelial cells (Ferrara *et al.*, 2003, Rini and Small, 2005). The VEGF gene is located on the short arm of chromosome 6 containing eight exons and may be differentially spliced to produce four mature isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) (Holmes and Zachary, 2005). Other isoforms were later recognized such as $VEGF_{145}$ and $VEGF_{183}$ (Tischer *et al.*, 1991). $VEGF_{165}$ is identified as the most predominant isoforms and is overly expressed in a variety of solid tumors. It is a potent angiogenic factor for tumor vascularization (Nishi *et al.*, 2005). VEGF-A exerts its biologic function by interacting with cell surface tyrosine kinase receptors consists of VEGFR-1/Flt-1 and VEGFR-2/Flk-1 expressed on the endothelial cells and neuropilin receptors (NP-1 and NP-2) expressed in the vascular endothelium (Dvorak, 2002). When VEGF-A binds its receptor, a number of downstream pathways are activated upon receptor dimerization and autophosphorylation of the intracellular tyrosine kinase domains (Ferrara *et al.*, 2003). VEGFR-2 was found to be the main receptor mediating the pro-angiogenic effects of VEGF-A (Ferrara *et al.*, 2003). Related molecules include VEGF-D and VEGF-C which are structurally similar, but are less homologous VEGF-A (Robinson and Stringer, 2001). These factors preferentially activate VEGFR-3 present predominantly on lymphatic EC and therefore promote lymphangiogenesis (Alitalo *et al.*, 2005). Experimental studies on mouse models have demonstrated that inhibiting the biological functions of VEGF-A with adenoviral delivery of a soluble form of VEGFR-1/2, by depletion of VEGF-A, and by the use of a chemical inhibitor of VEGF signaling impaired tumor angiogenesis and growth (Vajkoczy *et al.*, 1999, Inoue *et al.*, 2002, Casanovas *et al.*, 2005).

1.4.5 Interleukin-8 (IL-8)

Interleukin-8 is a member of the chemokine family, which is specific cytokines secreted by both normal cells and cancer cells. Generally, secretion of this cytokines is dependent on growth factors, inflammatory cytokines, and pathophysiological conditions (Koch *et al.*, 1992, Strieter *et al.*, 1992). IL-8 is known as a chemotactic protein secreted by

macrophages and activated monocytes that function to promote the directional migration of T lymphocytes, basophils, and neutrophils (Zlotnik and Yoshie, 2000). IL-8 possesses important functions in infectious diseases, autoimmune, and inflammatory (Koch *et al.*, 1992, Harada *et al.*, 1994). Due to strong inflammatory properties, IL-8 expression is controlled tightly with undetectable or low expression under normal circumstances. Over-expression of IL-8 has been demonstrated in a number of human cancers including those of melanoma, breast, and ovarian (Xu and Fidler, 2000, Freund *et al.*, 2003, Singh *et al.*, 2010). IL-8 plays a crucial role in angiogenesis in both physiologic and pathological conditions (Koch *et al.*, 1992, Strieter *et al.*, 1995a). Previous studies showed that IL-8 promoted tumor growth and increased metastatic potential of cancer cells (Miller *et al.*, 1998, Gomperts and Strieter, 2006). Additionally, IL-8 exhibited profound roles in tumor progression due to its ability to affect different cell of the tumor microenvironment (Xie, 2001, Waugh and Wilson, 2008).

(i) IL-8 structure

IL-8 is a small peptide (8 - 10 kDa), belonging to the CXC chemokine family and secreted by stromal (endothelial cells and fibroblasts) and tumor cells. The CXC chemokine family is further categorized based on conserved cysteines within the N-terminal amino acid sequence (Baggiolini *et al.*, 1997) as well as absence or presence of a three amino acid sequence, glutamic acid-leucine-arginine (ELR motif) which precedes the first cysteine residue in the CXC sequence. The ELR motif is important because the ELR (containing ELR+) CXC chemokines are potent promoters of angiogenesis and on the contrary, non-ELR (containing ELR−) CXC chemokines are anti-angiogenic (Strieter *et al.*, 1995a). Among these (ELR+) CXC chemokines, IL-8 is a potent angiogenic-promoting factor that stimulates formation of blood vessel in tumors. IL-8 gene is clustered on chromosome 4 between 4q12 and 4q21, consisting sequence identity between 24% to 46% with other CXC family members (Brat *et al.*, 2005). Transcription of the IL-8 gene produces a 99 amino acids protein which is processed through post-translational modification to yield two biological active IL-8 isoforms, with peptides of 72 and 77 amino acid residues (Hébert and Baker, 1993). The 72 amino acid

peptide is secreted by monocytes and macrophages. The 77 amino acid variant is the most abundant isoform of non-immune cells. The peptide structure of IL-8 is monomeric and composed of an NH2-terminal loop that is important for receptor activation, a central three anti-parallel beta sheet-strands connected by loops that provides a stable scaffold, and a C-terminal alpha helix that stabilizes the overall structure by folding over the small beta-sheet (Figure 18). IL-8 forms dimers although the monomer is a prevalent and active moiety of this chemokine (Rajarathnam *et al.*, 1994, Horcher *et al.*, 1998). Experimental data has demonstrated that IL-8 binds to its receptor N-domain as a monomer (Lowman *et al.*, 1997, Fernando *et al.*, 2004).

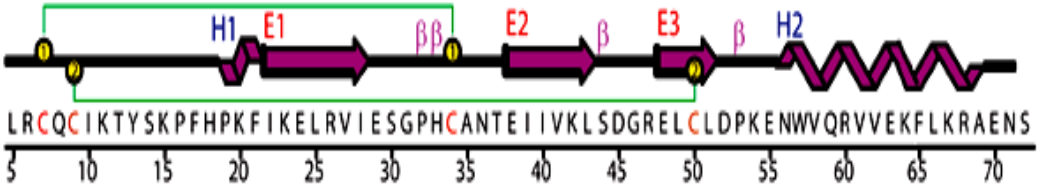


Figure 18: Primary structures of IL-8. Primary structure for interleukin-8 (PDB code: 3IL8). The N-terminal loop, which is responsible for receptor recognition, is restrained by two disulfide bonds. This is followed by a short turn of a 3_{10} helix (H1) that leads to a three-stranded anti-parallel β-sheet. The C-terminal α-helix folds over the β-sheet (H2) and helps to stabilize the overall tertiary structure (Kanagarajadurai and Sowdhamini, 2008).

The regulatory sequences have been categorized from -1 to -2500 bp of the 5'-flanking region of the IL-8 gene. One of the most characterized regions occurs at -425 to -70 bp and which possesses binding sites for the transcription factors C-EBP/NF-IL-6, AP-1, and NF-κB, as well as potential binding sites for the interferon regulatory factor-1, glucocorticoid receptor, and hepatocyte nuclear factor-1 (Mukaida *et al.*, 1994, Hoffmann *et al.*, 2002) (Figure 19). A recent study has identified a putative STAT3 binding site that positioned from -245 to -237 bp in the IL-8 promoter region (Oka *et al.*, 2010). Additionally, the estrogen response elements and C-EBP/NF-IL-6 consensus sites have been identified in the IL-8 promoter, and are involved in the activation of IL-8 production (Mukaida *et al.*, 1994,

Roebuck, 1999). NF-κB binding is required for promotion of IL-8 promoter activity (Matsusaka *et al.*, 1993). C-EBP/NF-IL-6 and AP-1 may contribute to transcriptional activity but dependent on the particular cell type (Mukaida *et al.*, 1990). Additionally, the expression of IL-8 is partially regulated by other stimuli such as environmental stresses (e.g., exposure to chemotherapy agents and hypoxia), chemical and steroid hormones, and inflammatory signals (e.g., tumor necrosis factor α) (Brat *et al.*, 2005).

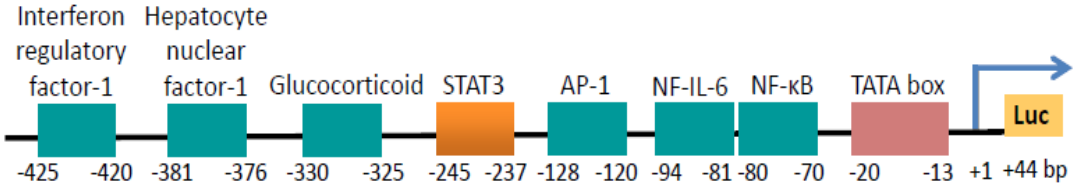


Figure 19: Binding sites for transcriptional factors in IL-8 promoter region. The consensus DNA-binding sites for known transcription factors in the promoter region of IL-8 gene. The putative STAT3 binding site is positioned from - 245 to -237 bp in the IL-8 promoter (Oka *et al.*, 2010). Adapted from Oka *et al*., 2010.

(ii) Roles of IL-8 and its cognate receptors in tumor microenvironment

The biological roles of IL-8 are produced by two different G protein–coupled receptors, termed as CXCR1 and CXCR2. IL-8 binds CXCR1 and/or CXCR2, with the same affinity, and thereby activates downstream signaling cascades that regulate cellular function (Murphy and Tiffany, 1991). CXCR1 and/or CXCR2 are known to be the only mammalian receptors of IL-8 with the specific amino acid sequence of ELR motif at the N-domain. CXCR1 receptor is activated solely in response to IL-8 but may also be activated by granulocyte chemotactic protein-2. Alternatively, activation of CXCR2 requires multiple CXC chemokines including neutrophil-activating peptide, growth-related oncogenes (GROα, β, and γ), and granulocyte chemotactic protein-2. They share 78% amino acid sequence homology and are also considered biologically active IL-8 cognate receptors (Ahuja and Murphy, 1996). The CXCR1 and CXCR2 receptors are highly expressed in neutrophils/tumor-associated macrophages, endothelial cells, and cancer cells. Multiple

reports have demonstrated that the over-expression of IL-8 by cancer cells was promoted by chemotherapeutic treatment or environmental stresses such as hypoxia. The increased synthesis and secretion of IL-8 from tumor cells exert profound effects on the tumor microenvironment (Figure 20). IL-8 secreted from cancer cells promotes cellular proliferation and survival. Additionally, IL-8 activates endothelial cells in tumor vasculature to stimulate *de novo* angiogenesis and promote chemotactic infiltration of neutrophils into the tumor. In addition to its capacity to promote cell invasion and migration, IL-8 also induces tumor-associated macrophages to secrete other growth factors to enhance cancer cell proliferation and invasion within the tumor (Waugh *et al.* 2008). Activation of IL-8 downstream signaling pathways include PI3K/Akt, Src-kinases and FAK, and MAPK signaling cascades, resulting in enhanced transcriptional activity of the signaling proteins implicated in angiogenesis, proliferation, migration and invasion (Todorović-Raković and Milovanović, 2013).

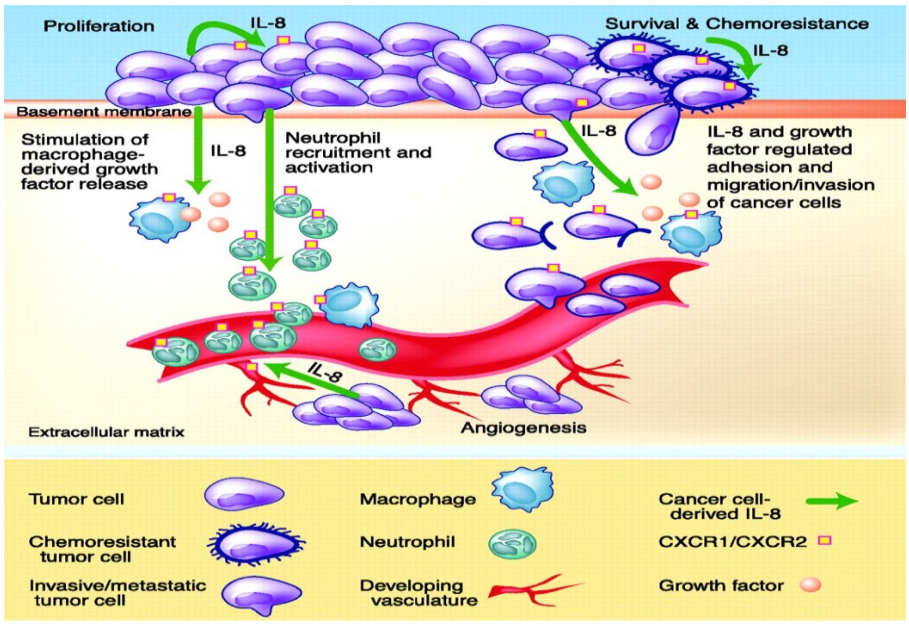


Figure 20: The role of IL-8 signaling in the tumor microenvironment. Tumor-derived IL-8 is able to exert profound effects on the tumor microenvironment. Secretion of IL-8 from cancer cells can enhance the proliferation and survival of cancer cells through autocrine signalling manner. In addition, tumor-derived IL-8 will activate endothelial cells in the tumor vasculature to promote angiogenesis and induce a chemotactic infiltration of neutrophils into the tumor site. IL-8 also stimulates tumor-associated macrophages to release growth factors for cancer cell migration and invasion (Waugh and Wilson, 2008). Reproduced with permission.

(iii) Biological effects of IL-8 in tumor angiogenesis and metastasis

IL-8 has an autocrine and/or paracrine tumor-promoting role in modulating the survival and proliferation of tumor cells. The biological activity of IL-8 in tumors and the tumor microenvironment may contribute to tumor progression through leukocyte infiltration, stimulation of cancer cell motility, angiogenesis, cancer cell growth and survival (Yuan *et al.*, 2005). Accordingly, Ewington *et al.* (2012) has recently demonstrated that the pathogenesis of endometrial carcinoma was positively associated with the expression of IL-8 and/or its cognate receptors (Ewington *et al.*, 2012). Additionally, Nastase *et al.* (2011) has suggested that IL-8 is useful to diagnose early stage colon cancer and to monitor the progression of colon tumors (Nastase *et al.*, 2011). The expression of IL-8 and IL-8 receptors in melanoma was shown to be positively correlated with disease progression (Singh *et al.*, 2010). Increased the expression of IL-8 and IL-8 receptors is associated with worse prognosis in breast cancer patients, indicating that the metastatic capacity of breast cancer cells was strongly correlated with the expression level of IL-8 (Miller *et al.*, 1998, De Larco *et al.*, 2001). Furthermore, a study has suggested that polymorphism of IL-8 and CXCR2 genes in breast cancer, which modulates the expression of IL-8 could promote tumor progression in breast cancer (Snoussi *et al.*, 2010). It has been reported that the effect of sex steroid in the regulation of IL-8 is contradicting and apparently varies in different tissues (Kanda and Watanabe, 2001, Bengtsson *et al.*, 2004). Increased IL-8 expression was observed in ER- breast cancer cells (MDA-MB-231) as compared to ER+ breast cancer cells (MCF-7) (Freund *et al.*, 2003, Lin *et al.*, 2004). Estrogen did not affect IL-8 expression in ER- or ER+ breast cancer cells, whereas it strongly induced TFF1 expression (a marker of estrogen responsiveness) in ER+ breast cancer cells. These observations support that the expression of IL-8 is associated with a higher invasiveness potential of cancer cells *in vitro* and therefore confirming the invasion-promoting effect of IL-8 (Freund *et al.*, 2003).

IL-8 plays a direct role in angiogenesis by promoting endothelial cell proliferation and survival as well as stimulating the expression of matrix metalloproteinase in endothelial

cells expressing CXCR1 and CXCR2 (Li *et al.*, 2003a). IL-8 stimulates endothelial proliferation and also capillary tube formation and these effects are inhibited by monoclonal antibodies to IL-8. A recent report has demonstrated that IL-8 stimulated VEGF A expression in endothelial cells via CXCR2, and hence increased the activation of VEGF receptors in an autocrine manner (Martin *et al.*, 2009). Moreover, IL-8 has been shown to enhance secretion of matrix metalloproteinase MMP-2 and MMP-9, suggesting that IL-8 can modulate invasiveness and/or ECM remodeling in the tumor microenvironment (Inoue *et al.*, 2000a, Inoue *et al.*, 2000b). As processes such as migration, cell proliferation, invasion, and angiogenesis are involved in metastasis, IL-8 expression by tumor cells can therefore have an effect on their metastatic capabilities. The expression of IL-8 is reported to correlate with the metastatic potential of cancer cells. The highly metastatic melanoma cells expressed higher level of IL-8 mRNA than melanoma cells with low metastatic capacity, suggesting that increased IL-8 mRNA expression is associated with the metastatic potential of cancer cells (Singh and Lokeshwar, 2009). Increased IL-8 in the tumor interstitium was shown to promote colon cancer growth and metastasis (Lee *et al.*, 2012). Conversely, the growth of colon cancer is prevented by the absence of its receptor CXCR2 in the tumor microenvironment (Lee *et al.*, 2012). In xenograft model, colon cancer cells expressing IL-8 formed significantly larger tumors with increased microvessel density when compared with the control cells (Ning *et al.*, 2011). Increased IL-8 expression promotes invasion and angiogenesis of breast cancer cells (Lin *et al.*, 2004). Additionally, over-expression of IL-8 in non-metastatic melanoma cells enhanced their invasion through Matrigel-coated filters and produced highly malignant and metastatic neoplasms in mice models (Kitadai *et al.*, 1999). When gastric carcinoma cells that secrete low levels of IL-8 protein were stably transfected with a IL-8 gene, the transfected cells produced more rapidly growing, highly vascular neoplasms as compared with the control cells (Kanayama *et al.*, 1999). Furthermore, treatment of HUVEC with conditioned medium from the IL-8 expressing cells stimulated HUVEC proliferation, suggesting that IL-8 is

pivotal in promoting endothelial cell proliferation and tumor vasculogenesis (Beierle *et al.*, 2001).

Accumulating clinical studies have reported that IL-8 expression to be associated with the advanced stages of cancers and therefore suppressing the effects of IL-8 has important clinical implications for tumor progression. Neutralizing antibodies to IL-8 receptors, humanized antibody against IL-8, small molecule IL-8 receptor inhibitor and repertaxin have already been employed in preclinical studies, where their ability to inhibit angiogenesis, tumor growth, and metastasis of various xenograft tumor models have been demonstrated (Ginestier *et al.*, 2010).

1.4.6 Lymphangiogenesis

Although both the blood and lymphatic systems have been implicated in metastasis, preclinical experimental data suggest that the majority of initial metastasis site is via the lymphatic (Stacker *et al.*, 2002, Sleeman and Thiele, 2009). Clinical evidence has also demonstrated that lymphatic vessel density, both in and around the tumors, was significantly associated with lymphatic metastasis and also worse survival outcomes (Ran *et al.*, 2010). Lymphangiogenesis is implicated in some of the physiological processes such as homeostasis, metabolism and immunity. In contrast, regional lymph node metastasis is common in cancer and is utilized as a marker for tumor dissemination, stage, and prognosis (Sundar and Ganesan, 2007). Migration of cancer cells to regional lymph nodes is enhanced by lymphangiogenesis, a process in which lymphatic vessels are generated from pre-existing lymphatics (Ran *et al.*, 2010). Lymphangiogenesis is a complex and coordinated process to induce proliferation, migration and tube formation as is observed in tumor angiogenesis (Sundar and Ganesan, 2007, Ran *et al.*, 2010). Lymphangiogenic factors are often expressed in tumor-infiltrating and stromal cells, promoting optional conditions for production of new lymphatic vessels. The most crucial lymphangiogenic factor that induces lymphangiogenesis is VEGFR-3, which is activated by VEGF-C and VEGF-D. Experimental studies have

demonstrated that VEGF-C/VEGF-D/VEGFR-3 signaling can promote lymphangiogenesis and dissemination from the primary tumor (Achen *et al.*, 2006). VEGFR3 expression is confined to lymphatic EC and facilitates the actions of VEGF-C and D (Shibuya and Claesson-Welsh, 2006). VEGF-C and -D are the most predominant growth factors in regulating this process (Nagy *et al.*, 2002, Bjorndahl *et al.*, 2005). Cancer cells potentially utilized the same chemokine ligands and its chemokine receptors for metastasis (Muller *et al.*, 2001). For example, human mammary carcinoma expressed chemokine receptors CXCR4 and CCR7, and their respective receptor CCL21 (secondary lymphoid chemokine) and CXCL12 (stromal-cell derived factor 1) are highly expressed in the target organs of metastasis (Muller *et al.*, 2001). Similarly, lymphatic EC synthesized chemokine CCL21, which activates CCR7 leading to chemoattraction and migration to lymph nodes. Breast cancer which expresses CXCR4 or CCR7 exhibit enhanced lymphatic metastasis as a consequence of chemoattraction and directional migration toward the ligand over-expressing lymph nodes (Ran *et al.*, 2010). Similar to angiogenesis, lymphangiogenesis promotes tumor metastasis.

1.4.7 Clinical implications on angiogenesis therapy

As angiogenesis is important and critical for cancer growth and metastasis, it is therefore a promising approach in controlling or limiting cancer progression (Weis and Cheresh, 2011). The association of the VEGF-A/VEGFR-2 signaling pathway as a pivotal regulator of tumor angiogenesis has resulted in generation of angiogenesis inhibitors against VEGF-A-mediated signal transduction. Some of the angiogenesis inhibitors have been approved for clinical use in cancer treatment. (Ellis and Hicklin, 2008).

Angiogenesis inhibitors can be defined as direct and indirect inhibitors. Direct angiogenesis inhibitors act directly on the tumor-derived endothelial cells by inhibiting their proliferation and migration. Indirect angiogenesis inhibitors are used to block the angiogenic factors and/or pathways that promote angiogenesis (Gasparini *et al.*, 2005). The indirect angiogenesis inhibitors that directly target VEGF-A, include Iressa (tyrosine kinase inhibitor),

Avastin or Bevacizumab (humanized anti-VEGF-A monoclonal antibody) and SU11248 (VEGF receptor blocking agent). In contrast, direct angiogenesis inhibitors abrogate the vascular endothelial cell proliferation, migration and/or resistance to apoptosis when in the presence of angiogenic proteins (Kerbel *et al.*, 2002). Angiogenic inhibitors such as endostatin also directly targets microvascular endothelial cells by preventing stimulation of angiogenesis-promoting factors (Kerbel *et al.*, 2002, Folkman, 2003). Depending on cancer type, treatment against angiogenesis may produce 3 - 6 months increase in patient progression-free survival. However, these angiogenic therapies may not provide enduring therapeutic efficacy, and confer only transitory clinical benefits to cancer patients. In these patients, revascularization may occur followed by enhanced metastasis (Baeriswyl and Christofori, 2009, Paez-Ribes *et al.*, 2009) (Figure 21). More importantly, inhibition of VEGF-A function may produce hypoxia-mediated increases in FGF expression to restart angiogenesis and tumor growth. Concomitant blockade of VEGF and FGF signaling, however, impeded the adaptive resistance to anti-angiogenic therapy of VEGF (Casanovas *et al.*, 2005). Hence, the emergence of resistance against anti-angiogenic therapies could be prevented by other angiogenic pathways stimulated as a compensatory mechanism (Hilberg *et al.*, 2008).

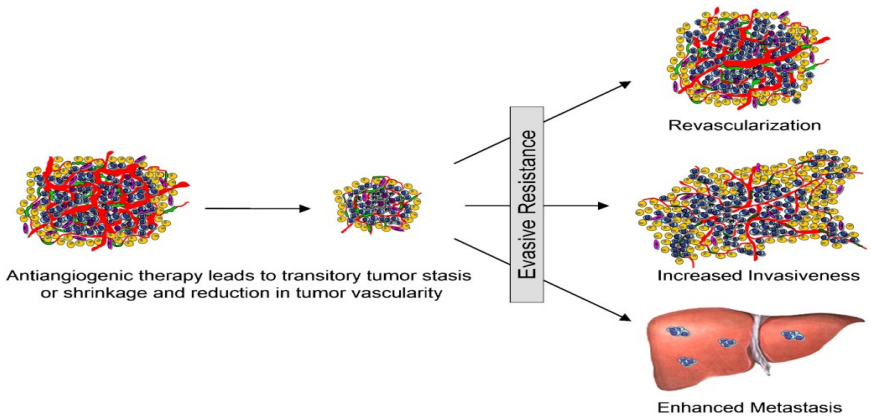


Figure 21: Adaptive resistance to anti-angiogenic therapies in cancers. Anti-angiogenic therapies produce transitory tumors shrinkage and reduction in tumor angiogenesis. Tumors subsequently are adapted to the angiogenic inhibitors. The emergent mechanisms of evasive resistance in tumors include revascularization via alternative pro-angiogenic signals, increased local invasiveness, and/or enhanced distant metastasis (Paez-Ribes *et al.*, 2009). Reproduced with permission.

1.5 Rationale and objectives of this study

Mounting evidence has shown that TFF3 protein is over-expressed in human mammary carcinoma where it is normally localized in mammary epithelial cells, suggesting that TFF3 exerts oncogenic effect on tumor growth and progression (May and Westley, 1997a, Poulsom *et al.*, 1997). TFF3 functions as a pro-survival and pro-invasive factor in mammary carcinoma (Emami *et al.*, 2001, Kannan *et al.*, 2010). Exogenous recombinant TFF3 induced HUVEC tubule formation *in vitro* (Rodrigues *et al.*, 2003b). The biological effects of TFF3 in the progression of mammary carcinoma have been extensively studied. However, the potential angiogenic effect of TFF3 has not been defined with the underlying mechanism is still not yet fully delineated. I propose that TFF3 promotes angiogenesis in mammary carcinoma and that underlying mechanism of TFF3 may involve modulation of targeted angiogenic factors and its transcription factors. The aims of this study are to investigate the stimulatory effects of TFF3 secreted from mammary carcinoma cells on the angiogenic activities of HUVEC as well as to delineate the mechanism of TFF3 in mammary carcinoma angiogenesis. The objectives of this study are:

1. To define the functional effects of TFF3 secreted from mammary carcinoma cells on the angiogenic behaviors of HUVEC;
2. To determine the *in vivo* angiogenic effect of TFF3 to stimulate microvessel density in mammary carcinoma cells-derived xenograft;
3. To identify the TFF3-stimulated angiogenic factors and the underlying mechanism of TFF3 in mammary carcinoma angiogenesis;
4. To determine the exogenous recombinant human TFF3 (rhTFF3) stimulation on the angiogenic behaviors of HUVEC; and
5. To define the functional effects of TFF3 secreted from HUVEC on the angiogenic behaviors of HUVEC

CHAPTER 2

Materials and Methods

2.1 Materials

The list of chemicals and reagents were enlisted in Table 1. The formulations and compositions of media, solutions, and buffers were also outlined in the Appendices. All water was purified through a Milli-Q purification system (Millipore Corporation, Billerica, MA, USA) and solutions were either autoclaved or filtrated through a 0.2 μm membrane.

Table 1: Chemicals and reagents

Chemicals and reagents	Source
100 bp or 1kb plus DNA ladder	Promega Corporation, Madison, WI, USA
10x Trypsin/EDTA	Gibco® Life Technologies, Carlsbad, CA
2x Laemmli Sample Buffer	Bio-Rad Laboratories, Inc., Hercules, CA, USA
3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)	Invitrogen Life Technologies, Carlsbad, CA, USA
4',6-diamidino-2-phenylindole (DAPI)	Vector Laboratories, Burlingame, USA
5-bromo-2-deoxyuridine (BrdU),	Calbiochem, Darmstadt, Germany
Acrylamide/Bis solution (37.5:1)	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Agarose (ultra pure)	Bio-Rad Laboratories, Inc., Hercules, CA, USA
AlamarBlue	Invitrogen Life Technologies, Carlsbad, CA, USA
Ammonium persulfate (APS)	Sigma Chemical Company, St Louis, MO, USA
Ampicillin	Sigma Chemical Company, St Louis, MO, USA
Anti-mouse CD31 (550274, BD)	BD Biosciences, Franklin lakes, NJ, USA
Anti-mouse IgG (BA-9200)	Vector Laboratories, Inc. Burlingame, CA, USA
Anti-mouse IgG, HRP-linked Antibody (Cat no. #7076)	Cell Signaling Technology Inc., Beverly, Massachusetts, USA
anti-rabbit IgG (BA-100)	Vector Laboratories, Inc. Burlingame, CA, USA

Anti-rabbit IgG, HRP-linked Antibody (Cat no. #7074)	Cell Signaling Technology Inc., Beverly, Massachusetts, USA
Anti-β-actin mouse monoclonal antibody (Cat no.C4: sc-47778)	Santa Cruz Biotechnology, Inc., Texas, USA
Bacto tryptone	BD Biosciences, Franklin lakes, NJ, USA
Bacto Yeast extract	BD Biosciences, Franklin lakes, NJ, USA
Blue/Orange Loading Dye, 6x	Promega Corporation, Madison, WI, USA
Boric acid	Sigma Chemical Company, St Louis, MO, USA
Bovine immunoglobulin	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Bovine serum albumin (BSA)	Sigma Chemical Company, St Louis, MO, USA
Bradford reagent	Bio-Rad laboratories, Inc., Hercules, CA, USA
Bromophenol Blue	Sigma Chemical Company, St Louis, MO, USA
Chloroform	Merck Group, Darmstadt, Germany
Protease inhibitor Cocktails	Roche Diagnostics GmbH, Mannheim, Germany
DC protein assay reagents	Bio-Rad laboratories, Inc., Hercules, CA, USA
DH5α™ strain of *Escherichia coli (E. coli)*	Invitrogen Life Technologies, Carlsband, CA, USA
Diethylpyrocarbonate	Sigma Chemical Company, St Louis, MO, USA
Dimethyl-sulphoxide (DMSO)	MP Biomedicals, CA, USA
Disodium Phosphate	Sigma Chemical Company, St Louis, MO, USA
Dithiothreitol (DTT)	Sigma Chemical Company, St Louis, MO, USA
Dual-Luciferase® Reporter (DLR™) Assay System	Promega Co., Madison, WI, USA
Endothelial cell basal medium supplemented with Bullet Kit (EBM™-2 SingleQuots)	Clonetics, Walkersville, MD
Ethanol (absolute, analytical grade)	Merck Group, Darmstadt, Germany
Ethidium bromide	Sigma Chemical Company, St Louis, MO, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma Chemical Company, St Louis, MO, USA
Fetal Bovine Serum (FBS)	Biowest Inc., Utah, USA

FuGENE 6 or FuGENE HD Transfection reagents	Promega Corporation, Madison, WI, USA
G418, Geneticin®	Sigma Chemical Company, St Louis, MO, USA
GelRed™	Biotium, Hayward, CA
Glycerol	Sigma Chemical Company, St Louis, MO, USA
Glycine	Bio-Rad laboratories, Inc., Hercules, CA, USA
Growth factor reduced Matrigel	BD Biosciences, Franklin lakes, NJ, USA
Hoechst 33258	Sigma Chemical Company, St Louis, MO, USA
Human IL-8 ELISA kit (ELISA MAX™ Deluxe Sets)	Biolegend, San Diego, CA
Hydrochloric acid	Sigma Chemical Company, St Louis, MO, USA
Hydrogen peroxide	Sigma Chemical Company, St Louis, MO, USA
Hygromycin B	Gibco® Life Technologies, Carlsbad, CA
Isopropanol	Merck Group, Darmstadt, Germany
L-glutamine	Gibco® Life Technologies, Carlsbad, CA
Lipofectamine® RNAiMAX	Invitrogen Life Technologies, Carlsbad, CA, USA
Magnesium Chloride	Sigma Chemical Company, St Louis, MO, USA
Mercaptoethanol	Sigma Chemical Company, St Louis, MO, USA
Methanol	Merck Group, Darmstadt, Germany
Mouse anti-BrdU	Roche Applied Science, Germany
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Invitrogen Life Technologies, Carlsband, CA, USA
N-lauryl sarcosine (Sarkosyl)	Sigma Chemical Company, St Louis, MO, USA
Paraformaldehyde	Sigma Chemical Company, St Louis, MO, USA
Penicillin (1000U/ml)	Gibco® Life Technologies, Carlsbad, CA
Phosphatase Inhibitor Cocktail	Sigma Chemical Company, St Louis, MO, USA
Platinum® PCR SuperMix High Fidelity	Invitrogen Life Technologies, Carlsbad, CA, USA
Podoplanin (ab11936, Abcam)	Abcam, Cambridge, United Kingdom

Potassium phosphate	Sigma Chemical Company, St Louis, MO, USA
Prestained protein ladder, ExtraPro Broad Range (10-245 kDa)	1st BASE Pte Ltd, Singapore
PureLink™ HiPure plasmid purification kit	Invitrogen Life Technologies, Carlsband, CA, USA
PVDF membrane	Bio-Rad laboratories, Inc., Hercules, CA, USA
Restriction enzymes and CutSmart™ Buffer	New England BioLabs®, MA, USA
RNeasy® Mini Kit	Qiagen, Limburg, Netherlands
RPMI 1640 medium	Nacalai Tesque, Inc., kyoto, Japan
SIGMA*FAST*™ 3,3′-Diaminobenzidine tablets	Sigma Chemical Company, St Louis, MO, USA
Sodium chloride	Sigma Chemical Company, St Louis, MO, USA
Sodium dodecyl sulfate (SDS)	Sigma Chemical Company, St Louis, MO, USA
Sodium hydroxide	Sigma Chemical Company, St Louis, MO, USA
Sodium Phosphate	Sigma Chemical Company, St Louis, MO, USA
Sodium Tetraborate	Sigma Chemical Company, St Louis, MO, USA
Streptomycin (1000 µg/mL)	Gibco® Life Technologies, Carlsbad, CA
SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase	Invitrogen Life Technologies, Carlsband, CA, USA
SuperScript® VILO™ cDNA Synthesis Kit	Invitrogen Life Technologies, Carlsbad, CA, USA
SuperSignal West Pico Chemiluminescent Substrate kit	Pierce Biotechnology, Inc., Rockford, Illinois, USA
SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, CA)	Invitrogen Life Technologies, Carlsband, CA, USA
T4 DNA ligase	Roche Diagnostics GmbH, Mannheim, Germany
Tetramethylethylenediamine (TEMED)	MP Biomedicals, CA, USA
Tris(hydroxymethyl) aminomethane (Tris)	Sigma Chemical Company, St Louis, MO, USA
Triton X-100	Sigma Chemical Company, St Louis, MO, USA

TRIzol reagent	Invitrogen Life Technologies, Carlsband, CA, USA
Trypan Blue	Gibco® Life Technologies, Carlsbad, CA
Tween-20	Sigma Chemical Company, St Louis, MO, USA
Vectashield Elite ABC kit standard (PK-6100)	Vector Laboratories, Inc. Burlingame, CA, USA
Vectastain® Elite ABC Kit	In Vitro technologies, NZ
Wizard® SV Gel and PCR Clean-Up System	Promega Corporation, Madison, WI, USA
X-ray film	Konica Minolta Inc., Tokyo, Japan

2.2 Methods

2.2.1 Human cell lines

The human mammary carcinoma cell lines, T47D and MCF-7 were obtained from the American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in RPMI 1640 media (Nacalai Tesque, Kyoto, Japan) supplemented with 100 µg/mL streptomycin, 100 IU/mL penicillin, 10% heat-inactivated fetal bovine serum, and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 incubator (Thermo Scientific, Waltham, MA). Human umbilical vein endothelial cells (HUVEC) (Clonetics Solingen, Germany) were a generous gift from Dr. Lim Yoon Pin, (Department of Biochemistry, National University of Singapore). HUVEC were cultured on a culture flask coated with 0.1% gelatin and were grown in endothelial cell basal medium (Clonetics, Walkersville, MD) supplemented with Bullet Kit (EBM™-2 SingleQuots), 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 µg/mL streptomycin at 37°C in a humidified 5% CO_2 incubator. HUVEC cultured from 4 - 8 passages were used for the experiments. Generation of MCF-7 and T47D cells with forced expression of TFF3 or depletion of TFF3 by siRNA have been previously described (Kannan *et al.*, 2010). The characteristics of the human mammary carcinoma cell lines and HUVEC are described in Table 2.

Table 2: Human cell lines description

Cell lines	Description
MCF-7	Mammary carcinoma cell line derived from a pleural effusion of an infiltrating ductal carcinoma (Dickson *et al.*, 1986). This cell line is well differentiated, epithelial, ER positive and non-invasive.
T47D	The T47D cell line is a ER+ mammary carcinoma cell line. It was isolated by Keydar *et al*. (1979) from a pleural effusion from a 54 year old female patient with an infiltrating ductal carcinoma of the breast. This differentiated epithelial substrain comprised cytoplasmic junctions and receptors for estrogen, other steroids and calcitonin.
HUVEC	Clonetics® Umbilical Vein Endothelial Cell (HUVEC) are tested positive for CD105 and CD31 endothelial markers, acetylated LDL uptake, von Willebrand factor VIII related antigen. It tested negative for alpha smooth muscle actin marker at passage four. HUVEC assayed for angiogenesis and vascular cell health markers.

2.2.2 Growth of human cell lines

The human cell lines were maintained using ATCC recommended conditions. Penicillin and streptomycin were also added to the media to prevent bacterial infection. Cells were grown in Greiner tissue culture treated flasks or plates at 37ºC in humidity 5% CO_2 incubator. All tissue culture work was undertaken in a laminar flow hood with sterile conditions.

2.2.3 Passaging and harvesting of human cell lines

All cell lines were grown in 75 cm^2 culture flasks containing approximately 20 mL of recommended growth media until cells were 85 - 95% confluent, after which a portion was passaged into a new flask to allow continuation of stock cultures. The confluent cells were then passaged by removing the media and subsequently rinsing the cells twice with PBS. A sufficient volume of 1x trypsin/EDTA (approximately 1 mL) to cover the surface of the cells

was added and the flask incubated at 37ºC in a humidified 5% CO_2 for two to five minutes. Cells were checked under the microscope to ensure that the cells had detached from the flask and most of them are individual cells. Following trypsinization, 5 - 8 mL of serum supplemented media was added to flask to neutralize the trypsin and the surface rinsed using a serological pipette. The cell suspensions were subsequently transferred to a 15 mL sterile Falcon tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cells pellet were resuspended in 10 mL of fresh serum-supplemented media. For maintaining of stock cultures, an appropriate cell number was seeded into a fresh culture flask and approximately 15 - 20 mL fresh media added, after which the cells were cultured at 37ºC in a humidified 5% CO_2 incubator. For cell counting, 20 µL of the cells suspension (total volume 10 mL media) was transferred to an Eppendorf tube and mixed with 20 µL of trypan blue dye (0.4%). Cell counts were then carried out using a haemocytometer. The number of cells contained in four quadrants of 16 squares was counted (twice). The counted squares were sum up and averaged, upon which the resulting number was used to determine the amount of cells/mL using the following formula:

Concentration of cells (cells /mL) = (Cells per 4 quadrants/4) x 10,000 x dilution factor

Total number of cells = Cells/mL x final volume

2.2.4 Storage of cell lines

Cells were trypsinized and resuspended in 10 mL media, counted and then centrifuged. Following removal of the supernatant, cells were resuspended in approximately 3 mL of ice cold freezing media. Aliquots of the cells suspension (1 mL) were transferred into cryogenic vial (Nalgene, Rochester, NY, USA). All vials were placed into an isopropanol containing freezing chamber (Nalgene, Rochester, NY, USA) and kept in a -80°C freezer for 24 hours to allow gradual cooling and freezing for cells preservation. The frozen cells were stored in the vapor phase of liquid nitrogen for long-term storage.

2.2.5 Revival of cell lines from liquid nitrogen storage

Cell aliquots from each cryogenic vial were thawed immediately in 10 mL of 37°C serum supplemented culture media and centrifuged at 1000 rpm for 5 min. The cells pellet was resuspended in 10 mL fresh serum supplemented culture media, transferred into a 25 cm^2 tissue culture flask, and cultured at 37°C incubator in humidified 5%CO_2. Media was changed the next day and every two days to allow growth of revived cells.

2.2.6 Transfection of mammalian cells

Transfection of specific DNA vectors was performed using FuGENE 6 (Promega, Madison, WI) as recommended by manufacture's instruction. Stable transfection required the additional selection of transfectants with appropriate antibiotics.

(i) Transient transfection:

Cells were plated at density of 400,000 cells per six-well plates one day before performing the transfection. When the cells reached 70 - 80% confluent, transient transfection was carried out to increase the transfection efficiency. For generation of mammary carcinoma cells with depletion of TFF3, mammary carcinoma cells were transiently transfected with a pSilencer 2.1-U6 hygro (Ambion, Austin, TX) expression vector that containing human TFF3 siRNA (Target DNA sequence: AAACAACGGTGCATAAATGAG) or control siRNA that encoding a siRNA comprises non-significant sequence similarity to human gene sequences. The expression vectors was constructed as previously described (Kannan *et al.*, 2010).

FuGENE 6 transient transfection was performed according to manufacturer's instruction. To prepare transfection solution required for 6-well plate, 12 µL of FuGENE 6 was diluted in 200 µL with serum free culture media and incubated for 10 minutes at room temperature. After that, 4 µg plasmid DNA was added into the diluted transfection reagent (FuGENE 6 reagent and plasmid DNA in serum free culture media at a 3:1 ratio). The FuGENE 6/DNA complex was incubated at room temperature for 20 minutes. The culture media was removed from cells and 1 mL of serum-free media was added to each well. The

FuGENE 6/DNA complex solution was added directly to the cells drop-wise. Following three to four hours incubation at 37ºC, 500 µL of complete media was applied to each well. The transfected cells were cultured at 37°C in humidified 5% CO_2 incubator for 24 hours or 48 hours prior performing any assays.

(ii) Stable transfection:

For stable transfection, cells were cultured to 70 - 80% confluence in a 75 cm^2 culture flask and stably transfected with FuGENE 6 (Promega, Madison, WI) according to the manufacturer's instructions. For generation of the stable cell line expressing TFF3, the MCF-7 or T47D cells were stable transfected with an pIRESneo3 expression vector containing the TFF3 cDNA (designated as MCF7-TFF3 or T47D-TFF3) or an empty pIRESneo3 vector as a control (designated as MCF7-Vec or T47D-TFF3). Pooled stable transfectants were selected with RPMI media, containing 800 µg/mL G418 for 21-28 days. Depending on the cell lines utilized, pooled stable transfectants were selected in varying concentration of Geneticin (G418). Initially when cell death was maximal, the media was changed every two days. Following that media was changed every three-four days. After three-four weeks, cells were trypsinized and transferred into a new 25 cm^2 culture flask. Stable expression was confirmed by semi-quantitative RT-PCR and Western blot analysis. Cell lines were then expanded and 10 vials of each cell lines were frozen. Cells were maintained in culture for a maximum of four months, following which a new vial was revived to avoid clonal selection.

2.3 Co-culture assays

In order to determine the functional effects of TFF3 secreted from mammary carcinoma on endothelial cells, an indirect co-culture transwell system was employed, in which mammary carcinoma cells were plated into the membrane of transwell insert (0.4 µm pore size polyester transwell insert) (Corning, NY, USA) co-cultured with HUVEC seeded in the bottom well of companion plate. Mammary carcinoma cells with forced expression of TFF3 or depletion of TFF3 co-cultured with HUVEC to determine the stimulatory effects of

TFF3 on HUVEC monolayer proliferation, cell cycle progression, survival, and tubule formation *in vitro*. To determine the effects of TFF3 secreted from mammary carcinoma cells on HUVEC migration and invasion, modified Boyden migration/invasion chamber assays using 8 μm transwell inserts (Greiner Bio-One GmbH, Germany) were conducted. Whereby, HUVEC were plated into the membrane of transwell insert co-cultured with mammary carcinoma cells seeded in the bottom well of companion plate.

2.3.1 RNAi transfection

The On-Target anti-IL-8 siRNA and control siRNA (non-targeted, random sequence) were purchased from Dharmacon (Chicago, IL) (Table 3). The Smartpool On-Targetplus Human IL-8 siRNA was a mixture of 4 SMART selection designed siRNA species targeting human IL-8 gene. Smartpool On-Target siRNAs designed to hybridize and destroy human IL-8 mRNA (Gen Bank Accession No. NM_000584). Subsequently, it silenced the human IL-8 gene or protein expression. The sequences of these Smartpool siRNAs were verified to be specific to human IL-8, thus eliminating the off-target effects. On-Targetplus non-targeting pool was a negative control siRNA with at least 4 mismatches to any rat, mouse, and human gene and microarray tested. The Smartpool On-Target siRNA and negative control were transfected into human mammary carcinoma cells using Lipofectamine® RNAiMAX Reagent (Invitrogen®, Carlsbad, CA, USA) according to manufacturer's instruction with slight modification. Briefly, mammary carcinoma cells were plated to be 70 - 80% confluent on a 6 well plate (400,000 cells/well) in complete media and incubated at 37^{o}C for 24 hours prior transfection. Final concentration of siRNA and negative control was 20 nM as recommended. 4 μL of Lipofectamine® RNAiMAX Reagent was diluted in 200 μL serum-free media. Similarly, 2 μL of siRNA and negative control was diluted in 200 μL serum-free media. Gently mixed the contents of each tube by pipetting carefully up and down and incubated at room temperature for 5 minutes. The diluted siRNA or negative control was added to respective tubes containing diluted Lipofectamine® RNAiMAX Reagent (1:1 ratio). Gently

mixed the contents of each tube by pipetting carefully up and down and incubated at room temperature for 20 minutes. The culture media was removed from cells and 1 mL of serum-free media was added to each well. The siRNA-lipid complex solution was then added directly to the cells drop-wise. Following three to four hours incubation at 37ºC, 500 µL of complete media was applied to each well. The transfected cells were cultured at 37^{o}C in humidified 5% CO_2 incubator for 24 hours or 48 hours prior performing assays. The silencing of IL-8 gene mRNA expression after 24 hours transfection was measured by semi-quantitative RT-PCR to identify the efficiency of siRNA. Additionally, conditioned medium from the cells were collected after 48 - 72 hours transfection and the silencing of IL-8 protein expression was measured by Human IL-8 ELISA kit (ELISA MAX^{TM} Deluxe Sets, Biolegend, San Diego, CA).

Table 3: The gene sequence of validated siRNAs and negative control

Smartpool On-Targetplus Human IL-8 siRNA, 10 nmol	**Target sequence**
On-Targetplus Smartpool siRNA J-004756-05, IL-8	GCAUAAAGACAUACUCCAA
On-Targetplus Smartpool siRNA J-004756-06, IL-8	GGACCACACUGCGCCCAACA
On-Targetplus Smartpool siRNA J-004756-07, IL-8	GCCAAGGAGUGCUAAAGAA
On-Targetplus Smartpool siRNA J-004756-08, IL-8	UGAAGAGGGCUGAGAAUUC
On-Targetplus Non-targeting pool, 5 nmol	

Source: Product datasheet from ThermoScientificBio.com/resource-library

2.3.2 Monolayer cell proliferation (Total cell number)

Mammary carcinoma cells with forced or depletion of TFF3 were trypsinized and seeded into six-well cell culture plates at a density of 50,000 cells/well. Cells were cultured in complete media for 24 hours at 37°C in 5% CO_2 incubator to ensure that they attached to the culture surface. Cell culture media was then changed into either 0.2% FBS or 10% FBS supplemented media. Assays were set up in triplicate and cells were counted every 24 hours

over two or three day periods. For HUVEC with forced or depleted expression of TFF3, counts were conducted over two or three days periods both for 0.2% FBS EBM or 10% FBS supplemented EBM medium, respectively. For co-culture assays, mammary carcinoma cells with forced or depleted expression of TFF3 (80,000 cells per transwell insert) were plated into the membrane of transwell insert (0.4 μm membrane pore size) (Corning Inc, NY, USA) in 10% FBS RPMI and incubated at 37°C for 24 hours after which the media was replaced with serum-free medium. HUVEC at density of 50,000 cells in serum-free EBM were seeded in bottom well of the companion plate. Assays were carried out for two to three days. Cells in each well were trypsinized with 0.5% trypsin and collected in 15 mL Falcon tubes. Cells were centrifuged at 1,000 rpm for 5 minutes to discard the supernatant and cell pellets was collected. Cells were then resuspended in 1 mL of supplemented media and counted using a haemocytometer.

2.3.3 5-bromo-2-deoxyuridine (BrdU) assay

Entry into S-phase of the cell cycle was determined by measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU) (Kaulsay *et al.*, 2001). BrdU labeling cells were trypsinized and seeded in triplicate into 24-well cell culture plates at a density of 200,000 – 250,000 cells per well. Cells were incubated overnight at 37°C in 5% CO_2 incubator, and after 12 hours the media was removed and the cells washed 3 times with PBS. Cells were then incubated in serum-free media for 18 - 20 hours. Co-culture experiments were performed as described for the total cell number assay. After 18 - 20 hours incubation at 37°C in 5% CO_2, the cells were pulse labeled with 20 μM BrdU (10 μL of 10 mM BrdU in 5 mL of media) was for 30 minutes at 37ºC in 5% CO_2. The media was discard and cells were washed 3 times with PBS. The procedure for detection of BrdU labeled cells is as below:

(i) Fixing

Cells were fixed by 4% formaldehyde diluted in PBS for 30 minutes at 4ºC and were washed 3 times with PBS. The endogenous peroxidase in the cells was blocked by 3% H_2O_2

in methanol. 1 mL of freshly prepared 3% H_2O_2 was added to each well and plates were incubated on a rocker at room temperature for 30 minutes. At the end of incubation, H_2O_2 was removed and wells were washed 3 times with PBS for 5 minutes each wash. Cells were then incubated with 2N HCl (1 mL per well) on a rocker at room temperature for 1 hour. After removing HCl, cells were washed 2 times with 0.1 M borate solution followed by a 5 minutes PBS wash.

(ii) Blocking and incubation with antibodies

Cells were incubated with 1 mL blocking solution (2% horse serum in PBS) per well for 1 hour at room temperature on a rocker and then washed 3 times with PBS for 5 minutes each wash. 1 mL of primary antibody at 1:100 dilution (mouse anti-BrdU antibody, Roche Applied Science, Germany) was added into the cells and incubated overnight at 4ºC. The Vectastain® Elite® ABC Kit (Vector Laboratories, Inc. Burlingame, CA, USA) was used in the following steps that included 2 hours incubation with 1 mL per well of secondary antibody solution at room temperature and 1 hour incubation with 1 mL per well of tertiary antibody solution at room temperature. All these incubations with different antibodies were performed with gentle agitation followed by 3 x 15 minutes washes with PBS.

(iii) Staining

The substrate solution was prepared by dissolving FAST 3,3-Diaminobenzidine hydrochloride (DAB) tablets (Sigma-Aldrich, MO, USA) in MilliQ water. 400 µL was added to each well and color development monitored. Staining was stopped with a PBS wash. Finally, cells were fixed in 70% cold ethanol at 4^{o}C for 30 minutes, washed twice with PBS, and stored at 4ºC until counted.

(iv) Determination of the BrdU labeling index

A total population of over 1000 cells was analyzed in ten arbitrarily chosen microscopic fields. Results were expressed as the percentage of cells synthesizing DNA (BrdU labeling index). Stained cells were counted using an OLYMPUS® IX71 Inverted Laboratory System Microscope, Olympus Optical Co., Tokyo, Japan.

2.3.4 Apoptosis assay

Bisbenzimides are cell-permeable, A-T specific dyes, which can intercalate in the minor groove of A-T regions thereby fluorescently labeling DNA. In this assay, one member of the bisbenzimides, Hoechst 33258, was employed. Apoptotic cell death was determined by fluorescent microscopic analysis of DNA staining patterns with Hoechst. Cells were plated at a density of 200,000 - 250,000 cells per well. After an overnight incubation at 37°C in 5% CO_2 in complete growth media to allow cells to attach, cells were treated with stimulation media (serum or serum-free; with or without drug treatment). Co-culture experiments were set up as described for the total cell number assay. After a culture period of 24 hours in serum-free media, media was carefully removed and the cells were fixed in 4% formaldehyde in PBS at 4^oC for 30 minutes. The cells were subsequently stained the karyophilic dye Hoechst 33258 (4 μg/mL Hoechst 33258, 1% Triton X-100 in PBS) at room temperature in the dark for 30 minutes. The staining solution was removed, washed twice with PBS, and culture plate was stored at 4°C. Apoptotic nuclear morphology was determined and examined under an inverted UV fluorescence microscope (Olympus). As compared to viable cells, the nuclear morphology of apoptotic cells characterized with nuclear fragmentation and condensation as well as the higher intensity of blue fluorescence of the nuclei. For statistical analysis, over 1,000 cells were counted in ten random microscopic fields at 200x magnification.

2.3.5 Invasion and migration assays

Migration and invasion assays were performed using modified Boyden chamber consisting cell culture transwell inserts (8 μm pores, Greiner Bio-One, Germany) and companion plates (24-well format) according to the manufacturer's instructions. For invasion assays, inserts were coated with growth factor reduced Matrigel (BD Bioscience) before plating the cells. Matrigel was diluted in serum-free media at 1/10 for MCF-7 and 1/50 for HUVEC respectively. HUVEC (30,000 cells) or mammary carcinoma cells with forced or

depleted expression of TFF3 (50,000 cells) in serum-free media was seeded into the membrane of transwell inserts and complete media was added to the bottom well of the companion plate. For co-culture assays, mammary carcinoma cells with forced or depleted expression of TFF3 (250,000 cells) in 10% FBS RPMI media were plated in the bottom well of the companion plate and incubated at 37^{o}C for 24 hours after which the media was replaced with serum-free media. A total of 30,000 HUVEC in serum-free EBM media were seeded into the membrane of transwell inserts. For migration and invasion assays using anti-IL-8 monoclonal antibody, mammary carcinoma cells with forced or depleted expression of TFF3 (250,000 cells) plated in 10% FBS RPMI media were plated in the bottom well of the companion and incubated at 37^{o}C for 24 hours after which the media was replaced with serum-free medium containing anti-IL-8 monoclonal antibody (50 μg/mL) and IgG control. A total of 30,000 HUVEC in serum-free media were seeded into the membrane of transwell inserts. For assays using exogenous human recombinant TFF3 (rhTFF3), different dilution of rhTFF3 in serum-free media and Bovine serum albumin (BSA) used as control were added to the bottom well of the companion plate. After 24 hours incubation at 37^{o}C, cells that had migrated or invaded to the lower surface of the transwell inserts were washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes, and stained with Hoechst 33258 (4 μg/mL Hoechst 33258, 1% Triton X-100 in PBS) at room temperature in the dark for 30 minutes. The staining solution was then discard, washed twice with PBS, and culture plate was stored at 4°C. The cells migrated to the lower surface of the entire transwell inserts were counted (OLYMPUS® IX71 Inverted Laboratory System Microscope, Olympus Optical Co., Tokyo, Japan).

2.3.6 Tubule formation *in vitro*

Growth factor reduced Matrigel (BD Bioscience) was thawed overnight in the fridge. The thawed Matrigel spread evenly over each well (300 μL) of a 24-well plate. The plates were incubated at 37ºC for 30 minutes in a cell culture incubator to allow the Matrigel to

solidify. A total of 30,000 HUVEC (30,000 cells/well for 24-well plate) in serum-free media were seeded in the Matrigel, incubated at 37^{o}C for 12 hours, and fixed with 4% paraformaldehyde at room temperature for 20 minutes. Tubules were visualized under the microscope at low magnification (x40). For co-culture experiments, mammary carcinoma cells with forced or depleted expression of TFF3 were plated at 50,000 cells per transwell inserts (0.4 μm pores) (Corning Inc, NY, USA) in 10% FBS for 24 hours after which the media was replaced with serum-free media. The mammary carcinoma cells plated into the transwell inserts co-cultured with HUVEC seeded in the Matrigel. The plate was incubated at 37^{o}C for 12 hours and tubules were visualized under the microscope at low magnification (x40). For data analysis, tubules formed by HUVEC in Matrigel of the entire transwell insert was photographed and analyzed by using ImageJ software (version 2.02; University of Texas Health Scientific Center at San Francisco).

2.3.7 Luciferase reporter assay

(i) Plasmids

Several plasmids such as pGL3-basic vector, pGL-IL-8-152 reporters were generous gifts from Prof. Takashi Tokino **(**Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University, Sapporo, Japan). The pGL3-basic vector was used as control plasmid. Fragment containing 5' flanking regions of the IL-8 gene were subcloned upstream of a luciferase reporter gene. pGL-IL8-152 reporter construct encompassing nucleotides -152 to +44 of the IL-8 promoter, which containing transcription factors, AP-1, NF-IL-6 and NFκB binding sites. The TATA box, and the NF-IL-6, NFκB and AP-1 binding sites are located at -13, -70, -80 and -120, respectively. Human IL-8 promoter reporter vector (full length, -4800 to + 104 bp) was a generous gift from Dr. Suswam A. Esther (Department of Neurology, University of Alabama in Birmingham, USA), and pRL-CMV control reporter vector (Promega, Madison, WI, USA) was used as an internal control for transfection efficiency of

luciferase reporter assay. The pcDNA 3.1 empty vector and pcDNA 3.1 expression vector containing human STAT3 siRNA have been previously described (Mohankumar *et al.*, 2008).

(ii) Luciferase reporter assay

Cells with forced or depleted expression of TFF3 were plated at a density of 200,000 cells per well in 12-well plate in triplicates and cultured in RPMI medium supplemented with 10% FBS at 37°C for 24 hours before transfection. Luciferase reporter assay was performed as previously described (Fan and Wood, 2007). Briefly, cells with forced expression of TFF3 or depletion of TFF3 transiently transfected with 2 µg of a reporter plasmids including human IL-8 promoter reporter vector (-4800 to + 104 bp) or pGL-IL8-152 reporter (-152 to +44 bp) and 40 ng pRL-CMV control reporter vector (Promega, Madison, WI, USA) using FuGENE 6 reagent according to the manufacturer's instructions. *Renilla* luciferase reporter vector used as control for transfection efficiency. For determination of IL-8 promoter activity in MCF7-Vec and MCF7-TFF3 with depletion of STAT3, MCF-7 cells with forced expression of TFF3 transiently transfected with 1 µg pcDNA3.1 empty vector containing control siRNA or pcDNA3.1 expression vector containing STAT3 siRNA, 1 µg human IL-8 promoter reporter (-4800 to + 104 bp), and 40 ng pRL-CMV control reporter vector (Promega, Madison, WI, USA) using FuGENE 6 reagent.

After 24 hours transient transfection, the reporter activities were measured by luciferase reporter assay using Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Madison, WI, USA). *Renilla* luciferase activity was used as an internal control. Briefly, 250 µL of 1x Passive Lysis Buffer (PLB) was added to each well of 12-well plate. Cells were incubated with PLB for 15 minutes on shaker and the cell lysate was then transferred to Eppendorf tubes. The cell lysate (20 µL) was added to each well of a white 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany). Luciferase Assay Buffer (50 µL) was dispensed into each well and mixed by tapping the plate gently. Firefly luciferase activity of the samples were measured by an Infinite 200 PRO micro plate reader (Tecan, Maennedorf, Switzerland), which was programmed to two sec measurement delay followed by a 10 second

measurement read for luciferase activity of samples. Subsequently, Stop & Glo® reagent (50 µL) was immediately added to each well mixed by tapping the plate gently. The *Renilla* luciferase activity was measured for normalization of transfection efficiency.

2.4 Biochemistry and molecular biology methods

2.4.1 IL-8 ELISA

A human IL-8 ELISA kit (ELISA MAX™ Deluxe Sets, Biolegend, San Diego, CA) was used to quantify the human IL-8 protein in conditioned medium of mammary carcinoma cells with forced expression of TFF3, depletion of TFF3, depletion of IL-8, and depletion of STAT3. Mammary carcinoma cells were grown to 70 - 80% confluence in a six-well plate. The cells were washed with PBS, pH 7.4 and the media was changed to serum-free for 24 hours. The media was collected after 24 hours and the ELISA assay was performed as per manufacturer's instructions. Briefly, the 96-well flat clear bottom of polystyrene microplate (Nunc™, Thermo Scientific) was coated with 100 µL diluted IL-8 specific monoclonal antibody and incubated overnight at 4°C. The plate washed 4 times with 300 µL Wash Buffer (0.05% Tween-20 in PBS, pH 7.4) per well and the residual buffer was blotted by firmly tapping plate upside down on absorbance paper. The plate was then blocked with 200 µL 1x Assay Diluent A to block non-specific binding and reduce background. The plated was then sealed with adhesive film and incubated for 1 hour at room temperature with shaking at 200 rpm on a plate shaker. While the plate is being blocked, the appropriate sample dilutions (1/100 dilution for conditioned media from HUVEC and conditioned media from mammary carcinoma cells can be used directly) and standards were prepared. A 1,000 µL of top standard recombinant human IL-8 at 1,000 pg/mL was prepared from stock solution (5ng/ mL) in 1x Assay Diluent A. Six two-fold serial dilutions of the 1,000 pg/mL top standard with 1x Assay Diluent A in separate tubes. After diluting, the human IL-8 standard concentrations were 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, and 15.6 pg/mL, respectively. 1x Assay Diluent A served as the zero standard (0 pg/mL). The plate

washed 4 times with 300 µL Wash Buffer. A total of 100 µL of standards and conditioned medium of samples were added to the appropriate wells. The plate was sealed with adhesive film and incubated at room temperature for 2 hours with shaking at 200 rpm on a plate shaker. The plate was then washed with 4 times with Wash Buffer. Biotinylated Detection Antibody diluted at 1:200 dilution in 1x Assay Diluent A was added into each well (100 µL/well), sealed the plate, and incubated at room temperature for 30 minutes with shaking. The plate was washed 4 times with Wash Buffer. Avidin-HRP diluted at 1:1,000 in 1x Assay Diluent A was added to each well (100 µL/well), sealed the plate, and incubated at room temperature for 30 minutes with shaking. The plate was then washed 5 times with Wash Buffer. For this final wash, soaked wells in Wash Buffer for 1 minute for each wash to minimize background noise. TMB Substrate Solution C was added into each well (100 µL/well) and incubated in the dark for 15 minutes. Positive wells turned blue color. The reaction was stopped by adding 100 µL of Stop Solution (2N H_2SO_4) to each well. Positive wells turned from blue to yellow. Absorbance reading was read at 450 nm within 30 minutes. The reference absorbance at 570 nm was subtracted from the absorbance at 450 nm. Results were expressed as concentration of IL-8 in the conditioned media (ng/mL).

2.4.2 RNA isolation

(i) RNA isolation using TRIzol reagent

Total RNA was extracted from exponentially growing cells (70% confluence) using TRIzol reagent. Cells were lysed by adding 1 mL TRIzol per 10 cm^2 culture flask. The homogenized sample was incubated at room temperature for 5 minutes. Next, 0.2 mL of chloroform per mL of TRIzol reagent was added to each tube and mixed vigorously for 15 seconds. After centrifugation at 12,000g for 15 minutes at 4ºC, the aqueous phase (RNA) was pipette out to a fresh tube and precipitated by adding 0.5 mL of 100% isoproponol per mL of TRIzol and incubated at room temperature for 10 minutes. A second centrifugation (at 12,000g for 15 minutes, 4ºC) was performed and the supernatant was discarded. The RNA

pellet was washed in 1 mL of 75% ethanol per mL TRIzol reagent used in the initial homogenization by centrifugation at 7,500 g for 5 minutes at 4ºC to remove salt. A third centrifugation was then performed at 7,500 g for 5 minutes at 4ºC and the pellet was air dried at room temperature and resuspended in RNase-free water or Diethylpyrocarbonate (DEPC) dH_2O. RNA sample was incubated in a water bath or heat block set at 55 - 60^oC for 10-15 minutes to increase the solubility of the RNA. The RNA sample can be used for downstream application or stored at -80^oC. The concentration and purity of RNA was determined spectrophotometrically using the ratios A260/A280 nm and A260/A230 nm. A ratio less than 1.8 may indicate the presence of contaminants within the sample.

(ii) RNA isolation using RNeasy® Mini Kit

Total RNA was isolated from exponentially growing cells (70% confluence) using RNeasy® Mini Kit (Qiagen, Limburg, Netherlands) as described by manufacturer's instruction. Briefly, cells were lysed by adding an appropriate volume of Buffer RLT containing either 1% β-mercaptoethanol or 2% 2M dithiothreitol (2M DTT), centrifuged at 12,000 rpm for 3 minutes, transferred the supernatant to a new microcentrifuge tube. Equal volume of 70% ethanol was added to the lysate (1:1 ratio), and mix well by pipetting. A total volume of 700 µL of the sample including any precipitate was transferred to an RNeasy Mini spin column placed in a 2 mL collection tube, centrifuged at 12,000 rpm for 1 minute, and flow-through was discarded. RNeasy spin column washed by adding 700 µL Buffer RW1, centrifuged at 12,000 rpm for 1 minute, and flow-through was discarded. After first washing, 500 µL Buffer RPE was added to the RNeasy spin column and centrifuged at 12,000 rpm for 1 minute. Addition of 500 µL Buffer RPE to the RNeasy spin column, centrifuged at 12,000 rpm for 2 minutes, and flow-through was discarded. The residual of buffer was removed out from the spin column by centrifuged at 12,000 rpm for 1 minute. The RNA sample was eluted by adding 30-50 µL RNase-free water directly to the spin column membrane and centrifuged at 12,000 rpm for 2 minutes. The concentration and purity of RNA was determined spectrophotometrically using the ratios A260/A280 nm and A260/A230 nm.

2.4.3 cDNA synthesis

Total RNA was converted to cDNA using SuperScript® VILO™ cDNA Synthesis Kit, which provides the high-temperature capability of SuperScript® III Reverse Transcriptase in an optimized conditions for generation of first-strand cDNA that commonly used in real-time quantitative RT-PCR (qPCR). For a single reaction, all components were combined in a tube on ice which included 5x VILO™ Reaction Mix (4 µL), 10x SuperScriptR Enzyme Mix (2 µL), RNA (up to 2.5 µg), DEPC-treated water to 20 µL reaction. The contents of the tube were gently mixed and reactions were incubated at 25ºC for 10 minutes and followed by 42ºC for 60 minutes. The reaction was terminated at 85ºC for 5 minutes. 1 µL (2 U) of *E. coli* RNase H was added and the tube was incubated at 37ºC for 20 minutes. The cDNA was stored at -20ºC until use.

2.4.4 PCR

Amplification of DNA templates was carried out using Platinum® PCR SuperMix High Fidelity (Invitrogen). The PCR mixture contains 22 U/ml *Pyrococcus* species *GB-D* thermostable polymerase, complexed recombinant *Taq* DNA polymerase and Platinum®, *Taq* Antibody in PCR buffers. For a single reaction, all components were combined in a reaction tube on ice (Table 4).

Table 4: Reaction components for PCR

Component	Volume
Platinum® PCR SuperMix High Fidelity	10.0 µL
DNA template (1-2 µg)	2.0 µL
Sense primer (10 µM)	1.0 µL
Anti-sense primer (10 µM)	1.0 µL
Autoclave MiliQ water	6.0 µL
Total reaction volume	20.0 µL

RT-PCR reactions were performed on a Peltier PCR Thermal Cycler (PTC-100, MJ research, Ramsey, MN). A negative control, including every component similar to normal reaction except template RNA, was included in each experiment. Each reaction started with two-step amplification program of initial denaturation at 95°C for 10 minutes, followed by PCR thermal cycle. The PCR cycling consisted of three steps including denaturation at 94°C for 30 seconds, annealing at specified temperature for 60 seconds, and extension at 72°C for 1 minute for the indicated cycles. The specific annealing temperature, number of cycles, and the primer sequences for individual reaction are listed in Table 6. Amplified β-actin served as an internal control for RNA quantity and quality. Amplified reactions were separated on a 1.5% agarose gel prepared in Tris-Boric acid-EDTA buffer (TBE 1x) containing of 1x GelRed™ (Biotium, Hayward, CA) or 1% ethidium bromide solution.

2.4.5 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed by using Invitrogen™ OneStep RT-PCR System with Platinum® *Taq* DNA polymerase Kit (Invitrogen). A master mix was prepared according to the following protocol to avoid localized differences in salt concentration (Table 7). Invitrogen™ OneStep RT-PCR Enzyme Mix was the last component added into the master mix In order to keep the maximum enzyme activity. The reaction components in master mix were enlisted as following (Table 5):

Table 5: Reaction components for semi-quantitative RT-PCR

Component	Volume
2x Reaction Mix	12.5 µL
Template RNA (0.01 pg - 1 µg)	variable
Sense primer (10 µM)	1 µL
Anti-sense primer (10 µM)	1 µL
SuperScript® III RT/ Platinum® *Taq* Mix	2 µL
Autoclave MiliQ water	top to 25 µL

RT-PCR reactions were performed on Peltier PCR Thermal Cycler (PTC-100, MJ research, Ramsey, MN). A negative control, including every component similar to normal reaction except template RNA, was included in each experiment. Each reaction started with 30 minutes reverse transcription at 50^{o}C followed by 15 minutes at 95^{o}C. The PCR cycling consisted of three steps including denaturation at 94^{o}C for 30 seconds, annealing at specified temperature for 30 seconds, and extension at 72^{o}C for 2 minutes for the indicated cycles. The specific annealing temperature, number of cycles, and the primer sequences for individual reaction are listed in Table 6. Amplified β-actin used as an internal control for RNA quantity and quality. Amplified reactions were separated on a 2% agarose gel prepared in Tris-Boric acid-EDTA buffer (TBE 1x) containing of 1x GelRed™ (Biotium, Hayward, CA) or 1% ethidium bromide solution. When the gel was polymerized, it was placed onto the GT agarose gel electrophoresis system that filled with 1x TBE buffer. A defined amount of RT-PCR product (approximately 10-20 µL) was mixed with 1.5 - 3.0 µL 6x DNA loading dye and then loaded into each lane of the agarose gel. A 100 bp or 1 Kb plus DNA ladder was used as a base pair marker. Electrophoresis was performed at 80 - 100 V for 20 - 40 minutes. RT-PCR products were visualized under UV light, photographed, and analyzed.

Table 6: Primers and PCR parameters for RT-PCR analysis

Gene	Primer sequence (5'-->3')	Product size (bp)	Annealing Temperature (^{o}C)	PCR cycles
VEGF-A	F: CATCACCATGCAGATTATGC R: AAAGTGCTCTGCGCAGAGTC	437 & 305	55	35
VEGF-B	F: GGATAGATGTGTATACTCGC R: TGTCTGGGTTGAGCTCTAAG	527 & 426	55	35
VEGFR1	F: GAGAGTATCACTCAGCGCAT R: GCAGTAAAATCCAAGTAACG	271	50	35
VEGFR2	F: TCCCGAGTTCTGGGCATTTC R: TGTACACGACTCCATGTTGG	451	50	35
IL-8	F: GAATGGGTTTGCTAGAATGTGATA R: CAGACTAGGGTTGCCAGATTTAAC	129	60	35
CXCR1	F: AGGGGCCACACCAACCTTCTG R:AGTGCCTGCCTCAATGTCTCCA	364	65	35
CXCR2	F: CAGTTACAGCTCTACCCTGCC R:CCAGGAGCAAGGACAGACCCC	451	65	35
STAT3	F: GATCCAGTCCGTGGAACCAT R:ATAGCCCATGATGATTTCAGCAA	75	60	35
TFF3	F: CTTGCTGTCCTCCAGCTCT R: CCGGTTGTTGCACTCCTT	125	60	35
β-ACTIN	F: ATGATATCGCCGCGCTCG R: CGCTCGGTGAGGATCTTCA	581	62	30

2.4.6 Real-time quantitative PCR

Total RNA was isolated from mammary carcinoma cells with forced expression of TFF3. Total RNA was then converted to cDNA by using SuperScript® VILO™ cDNA Synthesis (Invitrogen, CA) as described in section 2.4.3. Real-time qPCR was performed using the SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen, CA) following the manufacturer's protocol. The q-PCR experiment and data analysis were conducted by an ABI 7700® real-time PCR system (Applied Biosystems, USA). Multiple gene markers and three housekeeping genes (HKG) were utilized for real-time qPCR analysis. Real-time qPCR reaction mixture (20 μL) comprised of 12.5 μL SYBR® GreenER™ qPCR SuperMix, 2 μL of 200 nM primer (forward and reversed primers), and 3.5 μL RNase-free water was prepared. 5 ng of total cDNA (2 μL) was then added to the Real-time qPCR reaction mixture. Each

gene marker (Table 7) was added to a qPCR 365-well plate and run in triplicate. Real-time qPCR was performed using a two-step amplification program of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 20 seconds, 60°C for 30 seconds and 70°C for 30 seconds. At the end of the amplification, a melting curve analysis consisted of denaturation at 95°C for 1 minute and re-annealing at 55°C for 1 minute was performed. Standard curves were constructed from each experimental plate using serial 5-fold dilutions of untreated cDNA. The Ct-value for each reaction was calculated in order to obtain their geometric mean. The equation $E = 10^{(-1/slope)}$ was used to calculated the amplification efficiencies and ranged from 90 - 104% for all gene markers(Heid *et al.*, 1996). Melting curve analysis was used to ensure that there was no unspecific amplification or primer dimer in any of the reactions. Each experiment was carried out at least three times with independent samples. The gene expression of the gene marker was expressed as "fold change" and the gene expression of the gene marker in three repeated experiments were averaged (P value < 0.05 indicated statistically significant). Fold change > 2.0 indicated significant increased in mRNA level. The relative expression was calculated to compensate potential differences between markers, based on the efficiency (E), normalized by three housekeeping genes included β-actin, HPRT and GAPDH, and the Ct difference (Δ) of sample versus control ($\Delta Ct_{sample-control}$). Relative expression = $2^{-(Ct,Sample - Ct,HKG) - (Ct,Control - Ct,HKG)}$; Relative expression = 2^{-ddCt}. Changes in relative gene expression > 2.0-fold were taken as significant.

Table 7: Oligonucleotide primers of angiogenic markers for real time qPCR

Gene	Primer sequence (5'-->3')
VEGF-A	F: TACCTCCACCATGCCAAG R: GGTACTCCTGGAAGATGTC
IL-8	F: GAATGGGTTTGCTAGAATGTGATA R: CAGACTAGGGTTGCCAGATTTAAC
ANGPT1	F: CTCGCTGCCATTCTGACTCAC R: GACAGT TGCCATCGTGTTCTG
ANGPT2	F: TGGGATTTGGTAACCCTTCA R: GTAAGCCTCATTCCCTTCCC
COL18A1	F: TACCACTTCCCCAGCCTCTTCT R: TGCACC AGGTTCTGTGTAGAGC
TNF	F: CCAGGCAGTCAGATCATCTTCTC R: AGCTGGTTATCTCTCAGCTCCAC
TEK	F: GCTTGCTCCTTTCTGGAACTGT R: CGCCACCCAGAGGCAAT
TGFB1	F: GCCCTGGACACCAACTATTG R: CGTGTCCAGGCTCCAAATG
TGBS1	F: CTGCTCCAATGCCACAGTTC R: GGAGCCCTCACATCGGTTG
GADPH	F: TGCACCACCAACTGCTTAGC R: GGCATGGACTGTGGTCATGAG
HPRT	F: TGACACTGGCAAAACAATGCA R: GGTCCTTTTCACCAGCAAGCT
β-ACTIN	F: TTCCTGGGCATGGAGTC R: CAGGTCTTTGCGGATGTC

2.5 Plasmid DNA amplification

2.5.1 Bacterial strains

The *Escherichia coli* (*E.Coli*) strain DH5α^{TM} was obtained from Invitrogen (Groningen, The Netherlands). The genotype is DH5α: φ80dlacΔZM15, Δ(lacZY A-argF)U169, deoR, recA1, endA1, hsdR17(r-k, mk+), phoA, supE44, thi-1, gyr A96, rel A1 λ.

2.5.2 Growth of bacterial cultures

All bacterial cultures were grown aerobically in either liquid Luria-Bertani (LB) or on solid LB/agar plates at 37ºC. Depending on the specific experiments, as indicated in

appropriate sections, relevant antibiotics and additives were further added for selective growth.

2.5.3 Bacterial transformation of plasmids

Plasmids were amplified by transforming into a DH5α™ strain of *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Heat-shock competent DH5α™ bacteria were mixed with the plasmid and incubated on ice for 30 minutes. Transformation was performed using a heat shock method by placing the mixed bacteria and DNA in 42°C water bath (Grant Instruments Ltd., Cambridge, UK) for 2 minutes, immediately followed by cooling on ice for 5 minutes. LB Broth (1 mL) was added to each Eppendorf tube and incubated in the 37°C incubator (Sanyo Electric Co. Ltd., Japan) for 1 hour. Cultures were then spread on a LB agar plates. Due to the presence of a specific cassette present on the cloned plasmid (such as ampicillin in pIRES vector), transformed bacteria were resistant to the corresponding antibiotic and could be utilized as a selectable marker for positively transformed colonies. For instance, 100 μg/mL ampicillin is employed in selection of bacteria transformed with the pIRES plasmid. After bacterial plates were incubated in the 37°C incubator for 20 hours, a single colony from each petri dish was picked and placed in 200 mL of LB Broth containing 100 μg/mL ampicillin. Cultures were grown at 37°C with vigorous shaking (250 rpm) in a Bioline® automatic incubator shaker (Edwards Instrument Co., Narellan, NSW, Australia) for 20 hours.

2.5.4 Plasmid DNA purification

Bacterial transformation of plasmid DNA was initially performed by 20 hours incubation at 37°C with vigorous shaking in antibiotic-supplied LB broth. Next, extraction and purification of plasmid DNA from transformed DH5α™ bacteria was carried out using a PureLink™ HiPure plasmid purification kit (Invitrogen, CA). The bacterial cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4ºC using a TOMY MX-300 high speed refrigerated centrifuge (TOMY SEIKO CO., LTD, Japan). The pellet was collected and

resuspended in 10 mL chilled Suspension Buffer S1 with RNase by vortexing until homogenous, followed by addition of 10 mL Lysis Buffer (B7) and mixing thoroughly by gently inverting the capped Falcon tube 4-6 times until the lysate mixture was thoroughly homogenous. After 5 minutes incubation at room temperature, 10 mL Precipitation Buffer (N3) was immediately mixed with the lysate by inverting the tube until the mixture was thoroughly homogeneous. The mixture was then centrifuged at 15,000g for 10 minutes at room temperature. During this period, 30 mL of Equilibration Buffer (EQ1) was applied to equilibrate the PureLink™ HiPure Maxi Column. The DNA containing supernatant was loaded onto the equilibrated PureLink™ HiPure Maxi Column and allowed the solution in the column to drain by gravity. Columns were washed with 60 mL Wash Buffer (W8) and allowed the solution in the column to drain by gravity. Subsequently, the plasmid DNA was eluted with 15 mL Elution Buffer (E4). Isopropanol (10.5 mL) was mixed with the eluate at room temperature and centrifuged at 15,000g for 30 minutes at 4ºC to precipitate the DNA. The DNA pellet was washed with 5 mL of 70% ethanol at room temperature and then re-precipitated by centrifugation at 12,000 g for 10 minutes at 4ºC. The supernatant was carefully decanted and the pellet allowed to air dry before being redissolved in a suitable volume (50 - 200 μL) of ultrapure water. The concentration and purity of DNA measured using NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific) and determined spectrophotometrically by UV absorbance at 260 nm or ratios A260/A280 nm. The purified DNA was stored at -20ºC.

Small-scale plasmid DNA preparation was carried out using a Qiagen Miniprep Kit (Qiagen, Germany) according to the manufacturer's instructions. The 5 mL overnight bacteria culture was harvested by centrifugation at 16,000g for 15 minutes. The cell pellet was resuspended in Resuspension Solution, followed by the addition of Lysis Solution (0.2 M NaOH and 1% SDS) and Neutralization Solution. The lysate was centrifuged at 16,000 rpm for 10 minutes at room temperature, and the supernatant was transferred to a spin column. After spin for one minute, the column was washed by Washing Solution diluted with 95%

ethanol (162.8 mM potassium acetate and 27.1 mM Tris-HCl, pH 7.5). The column was centrifuged at 16,000 rpm for 1 minutes. The plasmid DNA was eluted in 50 µL ultrapure water.

2.5.5 DNA purification from agarose gel

DNA subjected to agarose gel electrophoresis, as described above, was visualized under UV light and the desired band excised with a sterile scalpel. The gel fragment was placed in a sterile Eppendorf tube and membrane binding solution from the Wizard® SV Gel and PCR Clean-Up System (Promega) was added (ratio of 10 µL solution per 10 mg of agarose gel slice). The mixture was vortexed and incubated at 50-60ºC until the gel was dissolved. DNA purification was processed by centrifugation. The gel mixture was transferred to the SV minicolumn and centrifuged for 1 minute at 16,000 g. The liquid was discarded and the column was washed 2 times for 25 minutes at 16,000 g. The SV column was carefully transferred to a clean microcentrifuge tube and 50 µL of nuclease free water was added. The tube was then centrifuged for 1 minute at 16,000g and the microcentrifuge tube containing the eluted DNA was stored at -20ºC.

2.6 Determination of protein expression

2.6.1 Protein extraction

Cells were plated in supplemented media at 60 - 70% confluence and incubated at 37^{o}C for 24 hours. Cells were washed once with ice cold PBS and harvested by mechanical scraping. The cells were resuspended in RIPA buffer (50 mM Tris-HCL pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1mM PMSF, 1 mM Na3VO4, 1x protease inhibitor) and incubated for 30 minutes on ice with vortexing every 5 minutes. From this step onwards, all procedures were performed on ice to avoid protein degradation. Lysates were centrifuged for 15 minutes at 16,000g and the supernatant collected. Lysis buffer (2% SDS, 20% glycerol, 60 mM Tris-HCl pH 6.8, 1x protease inhibitor) was used for proteins

containing a significant amount of disulfide bonds and subsequently harder to lyses. In this case, cells were washed three times with ice cold PBS for 10 minutes, dried and placed at -80ºC for one hour. 200 μL of lysis buffer was added to the petri dish and cells were harvested by mechanical scraping. Cell lysate was collected into an individual pre-chilled Eppendorf tube and sonicated in an 80T SONICLEAN benchtop ultrasonic cleaner (Soniclean Pty. Ltd., SA, Australia) for 1 minute. Finally, centrifugation was performed at 16,000g for 15 minutes at 4ºC. The supernatants were transferred to new Eppendorf tubes and stored at -80ºC or use immediately in subsequent assays. The protein concentration was measured in triplicates using DC protein assay (Bio-Rad).

2.6.2 Conditioned medium for concentration of protein

Cells were seeded in a 6-well plate at density of 200,000 cells in supplemented media and incubated at 37^{o}C for 24 hours or 48 hours. After which, media were changed to serum-free medium and incubated at 37^{o}C for 24 hours. Conditioned medium from the cells were collected and stored at -80^{o}C or proceed to ultrafiltration for concentration of protein using an Amicon® Ultra-0.5 centrifugal filter unit with a nominal molecular weight limit (NMWL) of 3 KDa (Milipore, Darmstadt, Germany). Sample recovery was 90% and molecular weight of the protein > 3 kDa retained by the membrane. Conditioned medium was loaded into the Amico® Ultra filter device and centrifuged at 14,000g for 15 minutes at 4^{o}C. To recover the concentrated solute, Amico® Ultra filter device was placed upside down in a clean microcentrifuge tube and centrifuged at 1,000g for 2 minutes in order to transfer the concentrated sample from the device to the tube. The protein concentration in the conditioned medium was determined by Bradford Assay.

2.6.3 Bradford assay

Bradford assay was used to determine the total protein concentration of a sample. This method is based on the proportional binding of the Coomassie dye to protein. Bovine

serum albumin (BSA) dissolved in water to a concentration of 4 mg/mL and a serial diluted of BSA standard solutions were prepared. Bradford reagent (Bio-Rad laboratories, USA) was diluted at a 1:5 ratio with water. 20 µL of the standards (in triplicates) were loaded into a 96-well microtitre plate (Greiner Bio-One, Germany). Solubilized protein samples were diluted 1:10 in RIPA buffer and 20 µL samples loaded in triplicates into the subsequent columns. Bradford reagent (200 µL) was added to each well. After incubation for 5 minutes at room temperature, absorbance of the standards and samples were read at 595 nm using an Infinite 200 PRO micro plate reader (Tecan, Maennedorf, Switzerland). The standard curve was plotted and absorbance of the unknown protein sample was calculated from the standard curve.

2.6.4 Bio-Rad DC protein assay

Working reagent A was prepared by adding 20 µL of reagent S to each mL of reagent A from the Bio-Rad Reagent package (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and mixing thoroughly. Standard used for Bio-Rad DC protein assay was BSA and 2 mg/mL BSA was diluted to make a series of standards containing from 0.2 mg/mL to 1.5 mg/mL protein with the RIPA buffer, which was the same buffer used for protein extraction. The standard curve was constructed from the serial diluted BSA in a range of 0 mg/mL to 1.5 mg/mL. Unknown protein samples can be used directly or diluted five times with RIPA buffer. Standards and unknown samples (5 µL) were transferred into a 96-well microtiter plate. 25 µL of working reagent (Reagent A + Reagent S) was then added into each well, followed by 200 µL Reagent B. The microtiter plate was incubated for 15 minutes with gentle agitation in the dark at room temperature. After removing any bubbles, absorbance readings were read at 750 nm using an Infinite 200 PRO microplate reader (Tecan, Maennedorf, Switzerland). Based on the absorbance of standards, the standard curve was plotted, and the absorbance of unknown protein sample was calculated from the standard curve.

2.6.5 Plate reading

The plate was placed onto an Infinite 200 PRO micro plate reader (Tecan, Maennedorf, Switzerland) with an appropriate absorbance wavelength setting. Standard curves were prepared by measuring the absorbance of BSA in a serial dilution. The calibration curves were linear ($r^2 > 0.99$) over the concentration range tested (0 – 2000 μg/mL) and the precision of all standards was reproducible with coefficient of variation, CV < 5%. The protein concentration was calculated by comparing the absorbance of the known protein BSA standards with those from the unknown samples.

2.6.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

A Laemmli SDS-polyacrylamide gel was cast using the Bio-Rad Mini-PROTEAN II system. The resolving gel was composed of 12% acrylamide (37.5: 1) in 0.375 M Tris-HCl, pH 8.8 and 0.1% SDS. The stacking gel was composed of 4% acrylamide in 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. The gels were polymerized by the addition of freshly prepared 0.1% ammonium persulfate and 0.01% Tetramethylethylenediamine (TEMED). Protein samples were mixed with an equal volume of 2x sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) (Bio-Rad) and heated at 100ºC for 10 minutes before being loaded into the gel. Electrophoresis was carried out in 1x running buffer (25 mM Tris, pH 8.3, 192 mM glycine and 0.1% SDS) at a constant voltage of 100 V for 2 hours. After electrophoresis was completed, the gel was removed from the gel cassette sandwich. After cutting off the stacking gel portion, the gel was equilibrated in transfer buffer for two minutes. Meanwhile, the Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), cut to the dimensions of the gel, was incubated in methanol for 1 minute and transferred into transfer buffer for another 3 minutes incubation. The filter paper and fiber pads were soaked in transfer buffer for 5 minutes. The electrophoresis transfer cassette was assembled in the following order: black side of the gel holder cassette; pre-wetted fiber pad; pre-wetted filter paper; gel; membrane; pre-wetted filter

paper; pre-wetted fiber pad; red side of the gel holder cassette. To optimize protein electrophoresis transfer, air bubbles were removed. The cassette was firmly closed and set in buffer tank filled with cold electrophoretic transfer buffer. The proteins separated by SDS-PAGE were transferred from the gels onto PVDF membrane for 2 hours at a constant voltage of 50 V.

2.6.7 Immunodetection of protein

Proteins were resolved in the SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 5% Bovine serum albumin in TBS with 0.1% Tween-20 (TBST) or non-fat dry milk in PBS with 0.1% Tween-20 (PBST) for 2 hours at room temperature. The blots were washed 4 times with PBST for 15 minutes and subsequently immunolabeled with the desired primary antibodies for 2 hours at room temperature or overnight at 4°C (Table 8). After the primary antibodies incubation, the blots were washed 4 times with PBST for 15 minutes and incubated with corresponding secondary Horseradish Peroxidase (HRP)-conjugated IgG antibody diluted in blocking buffer for 1 hour at room temperature. Following incubation, the blots were washed 4 times with PBST for 15 minutes before incubation with the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific) for 5 minutes in the dark. Blots were then exposed to X-ray film for 1 - 5 minute exposures and the film was developed by Konica SRX-101A processor (Konica Minolta Inc., Tokyo, Japan) for detection of protein band. For re-blotting, membranes were stripped for 5 minutes in stripping buffer (0.2 M NaOH). Blots were washed for 10 minutes with Milli-Q water followed by PBST at room temperature. Blots were blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBST) for 2 hour at room temperature. Thereafter, blots were immunolabeled with loading control such as β-actin monoclonal antibody. The procedure for immunodetection of the loading control was the same as described above. The recommended antibodies dilutions were listed in Table 10.

Table 8: Antibodies

Antibody	Supplier	Molecular weight (kDa)	Recommended dilution
VEGF-A	C-1: sc-7269, Santa Cruz Biotechnology	21/42	1:2000
TFF3	Anti-PGM 3 (animal 3105) polyclonal antibody was generated by Amiry *et al.* (2009) and synthesized by Biogenes, GmBH D, Berlin	7	1:2000
pSTAT3	B-7: sc-8058, Santa Cruz Biotechnology	86/91	1:2000
STAT3	C-20: sc-482, Santa Cruz Biotechnology	86/91	1:3000
IL-8	MAB-208, R&D systems	8	1:2000

2.5.8 Densitometric analysis of intensity of band

The amplified PCR products resolved in the agarose gel were captured by GelDoc imaging system (Bio-Rad) and intensity of the band of interest was analyzed by QuantityOne program (version 1.0.1, Bio-Rad). The protein bands developed in X-ray film were captured and analyzed by ImageJ. Bands of interest in the agarose gel or X-ray films were marked and their total integrated volumes were quantified against the background. Density was the corrected intensity integrated with volumes (in arbitrary units).

2.6 *In vivo* study

2.6.1 Angiogenesis *in vivo* assay

The angiogenesis *in vivo* assay using tumor xenograft models was performed in collaboration with Dr Tao Zhu (University of Science and Technology of China, Hefei). All the *in vivo* work related to mice models were performed under a standard protocol approved by the institutional animal care and use committee. MCF7-Vec and MCF7-TFF3 (5×10^6) were suspended in 100 µL Matrigel and injected into the first mammary (axillary) fat pad of six-week-old athymic (nu/nu) mice (Shanghai Slaccas Co.). Tumor xenografts produced by MCF7-Vec and MCF7-TFF3 were dissected and stored at -80°C for immunohistochemical (IHC) analysis and protein extraction. Tumor sections were prepared by fixing the tumor

tissues in 4% PFA (pH 7.4) overnight, embedded in paraffin, and cut into less than 5-μm thick paraffin (or frozen) sections. For IHC analysis, tissue sections of the tumors were mounted on glass slides, deparaffinised and rehydrated. The slides were rehydrated and incubated with 3% hydrogen peroxide for 30 minutes. The sections were blocked with 10% goat serum (Sigma) in PBS for 30 minutes and then incubated at room temperature for 90 minutes with the following antibodies: Anti-mouse CD31 (550274, BD) (1:100 dilution), anti-CD34/podoplanin (ab11936, abcam) (1:200 dilution), and anti-IL-8 antibody (R&D System) (1:100 dilution). The sections were washed with PBS and incubated with the respective biotinylated secondary antibodies such as anti-mouse IgG (BA-9200) or anti-rabbit IgG (BA-100) (1:300 dilution) at room temperature for 1 hour. Subsequently, an Elite ABC immunoperoxidase kit (PK-6100 vector Laboratory) was added to the sections and incubated for 30 minutes at room temperature, followed by visualization with diaminobenzidine (DAB) (ImmPACT DAB Substrate SK-4105 vector lab) (1 - 10 minutes at room temperature). Finally, the slides were mounted in DakoCytomation fluorescent mounting media and observed under a microscope (OLYMPUS AX70). Five different fields were chosen randomly from seven sections of each xenograft across the whole tumor. Photographs were captured with identical settings for all sections. Statistical analysis of protein expression was determined as previously described (Araneda *et al.*, 2005). A common threshold level was selected for all analyzed images to prevent intra-assay variations and the background level of the control sections was calculated for image analysis. ImageJ Program, version 1.45s (National Institute of Health, USA) was used to analyze the images (JPEG or TIFF files).

2.7 Bioinformatics Tools

2.7.1 Software for statistical analysis

The statistics software program Sigma Stat 3.1 was used in conjunction with Microsoft Excel for statistical analyses. The graphical presentations were generated using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). All experiments were performed for

three or four times. Numerical data were expressed as mean +/- SEM of triplicate determinants. Experiments were repeated for three times and data were analyzed by unpaired two-tailed student's *t* test or analysis of variance (ANOVA). A representative figure for each experiment is shown.

2.7.2 BLAST

Basic Local Alignment Search Tool (BLAST) was utilized for both DNA and protein sequence database searches (Altschul *et al.*, 1990). The basic algorithms of BLAST were used for gene identification searches, gene alignment searches, as well as transcription start site (TSS) estimations of various genes with uncharacterized promoter regions, specifically utilizing the EST-search function. BLAST was accessed via the National Centre for Biotechnology Information (NCBI). Website: http://www.ncbi.nlm.nih.gov/BLAST/.

CHAPTER 3

TFF3 Promotes *de novo* Angiogenesis in Mammary Carcinoma

3.1 Introduction

De novo angiogenesis is associated with tumor growth and metastasis (Schneider and Miller, 2005, Fox *et al.*, 2007, Paez-Ribes *et al.*, 2009). Adequate vascularization of the tumor is required for delivery of nutrients and oxygen to the growing tumor (Folkman, 1985, Risau, 1997). In a tumor microenvironment, the tumor is deprived of oxygen because of its distance from the nearest blood vessels and this hypoxic environment triggers the cancer cells to produce angiogenic factors or growth factors (Bergers and Benjamin, 2003, Gelao *et al.*, 2013). Angiogenic factors secreted from tumors or stromal cells stimulate neovascularization by activating endothelial cell proliferation, migration, survival, and degradation of cell to cell contact and cell to matrix adhesion (Gupta and Qin, 2003). Establishment of highly permeable and disorganized vasculatures in the tumor facilitates dissemination of cancer cells through the bloodstream to distant organs to form metastases (Baeriswyl and Christofori, 2009, Valastyan and Weinberg, 2011). Importantly, the survival and proliferation of the tumor depends on angiogenesis that is mediated by numerous of angiogenic factors, particularly vascular endothelial growth factors (VEGF) (Ferrara, 2004, Ferrara, 2005, Shibuya, 2008) and interleukin 8 (IL-8) (Koch *et al.*, 1992, Ning *et al.*, 2010).

A number of studies have reported that TFF3 expression was significantly associated with pro-survival and pro-invasive activities of mammary carcinoma cells (Emami *et al.*, 2001, Rodrigues *et al.*, 2003a, Kannan *et al.*, 2010, Ahmed *et al.*, 2012). Increased TFF3 expression in mammary carcinoma cells promoted cell proliferation, survival, oncogenicity, migration and invasion. (Kannan *et al.*, 2010, Ahmed *et al.*, 2012, Pandey *et al.*, 2014). TFF3 mRNA expression has been demonstrated to predict micrometastatic mammary carcinoma (Weigelt *et al.*, 2004, Mikhitarian *et al.*, 2005). The gene expression of TFF1 and TFF3 are associated with metastasis of breast cancer as reported by a microarray analysis of breast

cancer tissues (Smid *et al.*, 2006). Another recent studies displayed similar observations, increased TFF3 expression promotes metastasis and predicts poor survival outcomes of patients with mammary carcinoma (Kannan *et al.*, 2010, Pandey *et al.*, 2014). Inevitably, TFF3 may serve as an independent predictive biomarker for metastasis of mammary carcinoma.

In addition to its metastatic properties, TFF3 has recently been identified as a potential pro-angiogenic factor implicated in tumor angiogenesis. Immunohistochemical (IHC) analysis of tumor tissues has demonstrated that increased TFF3 expression was positively correlated with enhanced microvessel density (vessels labeled with CD31 marker) in gastric carcinoma (Dhar *et al.*, 2005) and breast carcinoma (Ahmed *et al.*, 2012). These observations were concordant with a study by Rodrigues *et al.* (2003b) who demonstrated that recombinant human TFF3 induced vessel formation in a chick chorioallantoic membrane (CAM) assay. However, the role of TFF3 in *de novo* angiogenesis in mammary carcinoma has not been determined.

IL-8 is a potent angiogenic factor involved in angiogenesis and metastasis of mammary carcinoma (Basolo *et al.*, 1993, Green *et al.*, 1997, Youngs *et al.*, 1997, De Larco *et al.*, 2001). It has been reported that elevated expression of IL-8 in mammary carcinoma cells was correlated with metastatic potential and tumor angiogenesis (Lin *et al.*, 2004). A clinical study has reported that triple negative (ER-PR-HER2-) breast cancer patients with elevated IL-8 expression were characterized by increasing invasiveness and metastatic potential that contributed to high risk of recurrence and poor prognosis (Rody *et al.*, 2011). The IL-8 serum concentration in patients with advanced breast cancer was higher than that of healthy subjects. Increased IL-8 expression was associated with worse survival outcome, a higher tumor load, and involvement of liver or lymph node (Benoy *et al.*, 2004). The presence of bone metastasis in patients with advanced breast cancer was positively correlated with increased IL-8 expression (Simeone *et al.*, 2007), suggesting that IL-8 is an independent

predictive marker for breast cancer patients with high metastatic capacity and poor survival outcome (De Larco *et al.*, 2003, Simeone *et al.*, 2007).

It has also been shown that IL-8 and its cognate receptors namely CXCR1 and CXCR2 were positively associated with poor prognosis in patients with breast cancer (Miller *et al.*, 1998, Benoy *et al.*, 2004). The ability of IL-8 to elicit angiogenic activity depends on the expression of its receptors by endothelial cells (Singh *et al.*, 2010). IL-8 stimulates endothelial cell proliferation and capillary tube formation in a concentration-dependent manner, and these angiogenic effects can be abrogated by monoclonal antibodies inhibitory to IL-8 (Heidemann *et al.*, 2003, Li *et al.*, 2005). Neutralizing antibodies to CXCR1 and CXCR2 inhibit migration of endothelial cells promoted by IL-8, suggesting that these two receptors are critical for activation of the angiogenic responses of IL-8 (Salcedo *et al.*, 2000a, Li *et al.*, 2005). Tumor cells exploit the expression of IL-8 and its receptors to enhance tumor progression through regulation tumor growth, angiogenesis, and immune response. Increased Il-8 expression alters the tumor microenvironment to be favorable for cancer cells to metastasize and colonize at the targeted organs (Yuan *et al.*, 2005, Singh *et al.*, 2010, Lee *et al.*, 2012). Inhibition of IL-8 by humanized anti-IL-8 antibodies attenuated tumor growth, angiogenesis and metastasis of human melanoma (Huang *et al.*, 2002). Administration of a humanized monoclonal antibody against IL-8 has been shown to suppress the growth of bladder cancer in xenograft models (Mian *et al.*, 2003).

IL-8 signaling activated growth factor receptors and stimulated the activity of MAPK signaling cascade, which is related to the promoting effect of IL-8 on cell proliferation and survival of cancer (Brew *et al.*, 2000, Luppi *et al.*, 2007) and endothelial cell lines (Li *et al.*, 2003a, Waugh and Wilson, 2008). It is known that PI3K is involved in the regulation of IL-8. Activation of PI3K/Akt signaling pathway is modulated by IL-8 to promote cell survival, angiogenesis, and migration of mammary carcinoma cells (Cheng *et al.*, 2008, Waugh and Wilson, 2008, Todorović-Raković and Milovanović, 2013). IL-8 signaling is likely to promote the transcriptional activity of multiple genes that involved in angiogenesis, migration,

invasion and the evasion of apoptosis (Waugh and Wilson, 2008). In addition to IL-8, VEGF-A and TFF3 were connected to signal transducers and activators of transcription 3 (STAT3) signaling as these two peptides promote activation of STAT3 by tyrosine phosphorylation at 705 amino acid residues (Rivat *et al.*, 2005, Pandey *et al.*, 2014). As a result, the expression of TFF3 and VEGF-A was up-regulated by STAT3 (Niu *et al.*, 2002, Tebbutt *et al.*, 2002). The persistent STAT3 activity enhanced by growth factors as well as additional oncogenes which promote oncogenic transformation and invasion of cancer cells such as Src family kinases and RhoA (Turkson *et al.*, 1998, Simon *et al.*, 2000, Goiot *et al.*, 2001). The activated dimeric STAT3 translocates into the nucleus, where it binds to the targeted DNA response elements within the promoter region of a specific gene and subsequently activate its gene transcription (Horvath *et al.*, 1995, Darnell, 1997). TFF peptides directly induced cell scattering and cellular invasion of kidney and colonic carcinoma cells through autocrine loops (Emami *et al.*, 2001). Alternatively, the pro-invasive activity of TFF3 was controlled by activation of Src and Rho-dependent signaling pathways that led to tyrosine phosphorylation of STAT3, suggesting that STAT3 may be a crucial transcription factor involved in the mechanistic action of TFF peptides (Emami *et al.*, 2001). A recent study observed that TFF3 expression in mammary carcinoma cells stimulated tyrosine phosphorylation of c-Src, which subsequently increased STAT3 activity and led to the down-regulation of E-cadherin to promote cell invasion (Pandey *et al.*, 2014). Blockade of STAT3 signaling by a dominant-negative element (STAT3β), depletion of STAT3 by siRNA and inhibition of STAT3 activation by STAT3 inhibitory peptide abrogated cellular invasion and growth of colon cancer in xenograft models (Rivat *et al.*, 2005). Inhibition of STAT3 activity using STAT3 inhibitors and depletion of STAT3 by siRNA largely inhibited the ability of TFF3 to stimulate invasion of mammary carcinoma cells (Pandey *et al.*, 2014).

The persistent STAT3 activity exhibits anti-apoptotic, mitogenic, and angiogenic effects in cancer cells. STAT3 stimulates tumor angiogenesis by increasing the promoter activity of VEGF-A (Bromberg *et al.*, 1999, Shen *et al.*, 2001, Niu *et al.*, 2002). Furthermore,

STAT3 has been suggested as a regulator of IL-8 at the transcriptional level by direct binding to the IL-8 promoter (Oka *et al.*, 2010). However, the underlying mechanism by which TFF3 promote *de novo* angiogenesis in mammary carcinoma has not yet been determined. The role of STAT3 in TFF3-stimulated angiogenesis in mammary carcinoma remains elusive. I postulate that a similar STAT3 signaling pathway between TFF3 and the TFF3-stimulated pro-angiogenic factor, which can be a target gene of STAT3 and coordinately mediates TFF3 to promote angiogenesis in mammary carcinoma. Herein, I have determined the effect of TFF3 secreted from mammary carcinoma cells on the angiogenic behaviors of endothelial cells to promote *de novo* angiogenesis in mammary carcinoma and delineated the underlying mechanism by which TFF3 promotes *de novo* angiogenesis in mammary carcinoma.

In this study, I utilized two human mammary adenocarcinoma cell lines, namely MCF-7 and T47D cells, as *in vitro* models, by stable forced expression of TFF3 or depletion of TFF3 in these cell lines. These cell lines are easily propagated and are amendable to genetic manipulation (Masters, 2000). Additionally, these cell lines can be implanted into immunodeficient mice as xenograft models and the effects of altered gene function and pharmacological inhibition of tumorigenicity in breast cancer both *in vitro* and *in vivo* can be determined (Lacroix and Leclercq, 2004). Additionally, the effect of TFF3 secreted from mammary carcinoma cells on endothelial cells was determined by using an indirect co-culture transwell system, in which mammary carcinoma cells plated on the membrane of transwell inserts were co-cultured with endothelial cells seeded in the bottom well of the companion plate. This indirect co-culture system enables two different type of cells to culture together, which is mimic to the *in vivo* situation and allows the soluble factors from mammary carcinoma cells to diffuse through the pore of the membrane to the endothelial cells and subsequently modulate the angiogenic behaviors of endothelial cells. To examine the angiogenic potential of TFF3, a tubule formation assay was performed in which mammary carcinoma cells plated on the membrane of transwell inserts co-cultured with endothelial cells seeded in the Matrigel coated in the bottom well of the companion plate. Tubules formed by

HUVEC in the Matrigel can be quantified to assess the angiogenic activity of TFF3 on the endothelial cells (Bishop *et al.*, 1999) and the morphology of tubules generated by endothelial cells is representative of capillary vessel formation *in vivo* (Donovan *et al.*, 2001, Bagley *et al.*, 2003). By using this approach, the roles of TFF3 secreted from mammary carcinoma cells on the angiogenic activities of HUVEC can be determined.

3.2 Results

3.2.1 Expression of TFF3 mRNA in mammary carcinoma cells

The expression of TFF3 mRNA in several ER+ mammary carcinoma cell lines (MCF-7, T47D, MDA-MB-361, and BT474) and one ER- mammary carcinoma cell line (MDA-MB-231) were analyzed by semi-quantitative RT-PCR. ER+ mammary carcinoma cell lines expressed higher TFF3 mRNA as compared to the ER- mammary carcinoma cell line (MDA-MB-231) (Figure 22A). The expression of TFF3 in mammary carcinoma cell lines was consistent with the previous studies reported that TFF3 was associated with ER status (May and Westley, 1997a, Doane *et al.*, 2006, Pandey *et al.*, 2014). MCF-7 and T47D cells expressed moderate levels of TFF3 mRNA were chosen to be utilized as mammary carcinoma cell models (Kannan *et al.*, 2010, Pandey *et al.*, 2014).

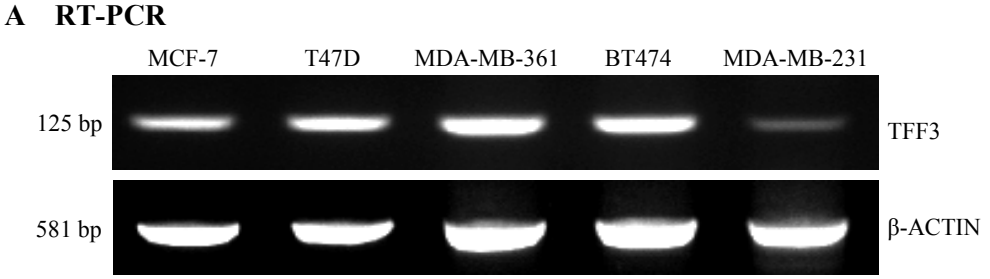


Figure 22: TFF3 mRNA expression in several of mammary carcinoma cell lines. A, Semi-quantitative RT-PCR analysis of TFF3 mRNA expression in several mammary carcinoma cell lines including MCF-7, T47D, MDA-MB-316, and BT474 (ER+ mammary carcinoma cells) and MDA-MB-231 (ER- mammary carcinoma cells). β-ACTIN was used as a loading control in semi-quantitative RT-PCR and Western blot analysis.

3.2.2 Forced expression of TFF3 in mammary carcinoma cells increased TFF3 expression

To determine the stimulatory effect of TFF3 secreted from human mammary carcinoma cells on the angiogenic behavior of HUVEC, MCF-7 and T47D cells were stable transfected with a pIRESneo3 expression vector containing TFF3 cDNA (designated as MCF7-TFF3 and T47D-TFF3) or a pIRESneo3 empty vector (designated as MCF7-Vec and T47D-Vec). The expression of TFF3 mRNA and protein in MCF-7 and T47D with forced expression of TFF3 was verified by semi-quantitative RT-PCR and Western blot analyses. Forced expression of TFF3 in MCF-7 and T47D cells increased TFF3 mRNA (Figure 23A-B) and TFF3 protein (Figure 23C-D) when compared with the respective control MCF7-Vec and T47D-Vec.

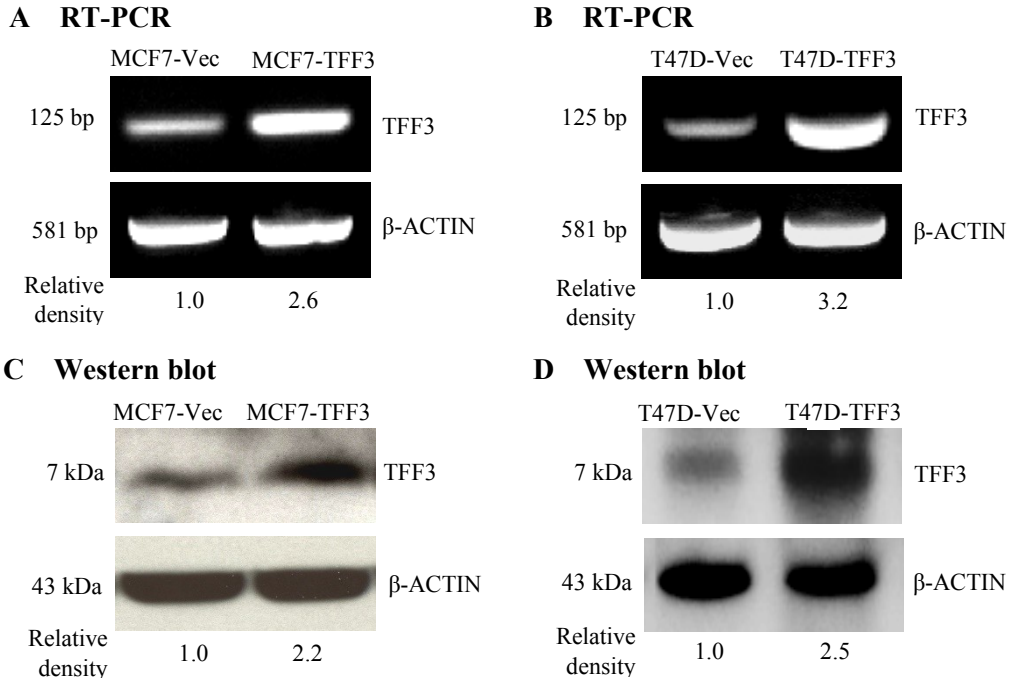


Figure 23: Forced expression of TFF3 in mammary carcinoma cells increased TFF3 expression. A-B, semi-quantitative RT-PCR analysis of TFF3 mRNA in MCF-7 and T47D cells with forced expression of TFF3. C-D, Western blot analysis of TFF3 protein in MCF-7 and T47D cells with forced expression of TFF3. β-ACTIN was used as a loading control in both semi-quantitative RT-PCR and Western blot analysis.

3.2.3 Forced expression of TFF3 in mammary carcinoma cells promoted HUVEC monolayer proliferation, cell cycle progression, and survival

A previous study has reported that TFF3 secreted from human mammary carcinoma cells is oncogenic and promoted proliferation of mammary carcinoma cells *in vitro* and increased tumor growth in xenograft models *in vivo* (Kannan *et al.*, 2010). Therefore, the stimulatory effect of TFF3 secreted from mammary carcinoma cells on the angiogenic activities of HUVEC was determined by using an indirect co-culture system, in which MCF7-TFF3 and T47D-TFF3 were plated on the membrane of the transwell inserts (0.4 μm membrane pores) and co-cultured with HUVEC seeded in the bottom well of the companion plate. Co-culturing HUVEC with MCF7-TFF3 and T47D-TFF3 increased HUVEC monolayer proliferation in both 10% FBS conditions for 72 hours and 0.2% FBS conditions for 48 hours, when compared with HUVEC co-cultured with respective control MCF7-Vec and T47D-Vec. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 increased HUVEC monolayer proliferation by 35% and 40%, respectively after 72 hours when compared with the respective control cells (Figure 24A-B). TFF3 secreted from MCF7-TFF3 and T47D-TFF3 promoted a small increase of HUVEC monolayer proliferation by 12% and 13%, respectively after 48 hours when compared with the respective control cells (Figure 24C-D). TFF3 secreted from mammary carcinoma cells promoted HUVEC monolayer proliferation.

Endothelial cell proliferation and survival are the most essential components to support and maintain vasculature in the tumor (Nor *et al.*, 1999). Increased monolayer cell proliferation is achieved by increased cell cycle progression and/or decreased apoptotic cell death. The effect of TFF3 secreted from mammary carcinoma cells on HUVEC cell cycle progression and apoptotic cell death was determined by co-culturing HUVEC with MCF7-TFF3 and T47D-TFF3. Cell cycle progression was analyzed by 5-bromo-2-deoxyuridine (BrdU) incorporation assay. BrdU labeling is as a marker of DNA synthesis. BrdU can be incorporated into nuclear DNA during the S-phase of the cell cycle as a substitute for thymidine during DNA replication (Miller and Nowakowski, 1988). TFF3 secreted from

MCF7-TFF3 and T47D-TFF3 increased HUVEC cell cycle progression in serum-free conditions by 28% and 21%, respectively when compared with the respective control cells. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 significantly increased HUVEC proliferation in 10% FBS conditions by 43% and 21%, respectively when compared with the respective control cells (Figure 25A-B). TFF3 secreted from MCF-7 and T47D with forced expression of TFF3 promoted HUVEC cell cycle proliferation in both serum-free and 10% FBS conditions.

In addition to cell proliferation, resistance to apoptosis is the other essential component for the survival of endothelial cells. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 significantly decreased HUVEC apoptotic cell death by 52% and 39%, respectively when compared with the respective control cells in serum-free conditions. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 decreased HUVEC apoptotic cell death by 61% and 50% respectively when compared with the respective control cells in 10% FBS conditions (Figure 25C-D). TFF3 secreted from mammary carcinoma cells inhibited HUVEC apoptotic cell death in both serum-free and 10% FBS conditions. Additionally, TFF3 secreted from mammary carcinoma cells protected HUVEC from apoptosis induced by serum starvation. Therefore, TFF3 secreted from mammary carcinoma cells promoted proliferation of HUVEC by increased cell cycle progression and decreased apoptotic cell death.

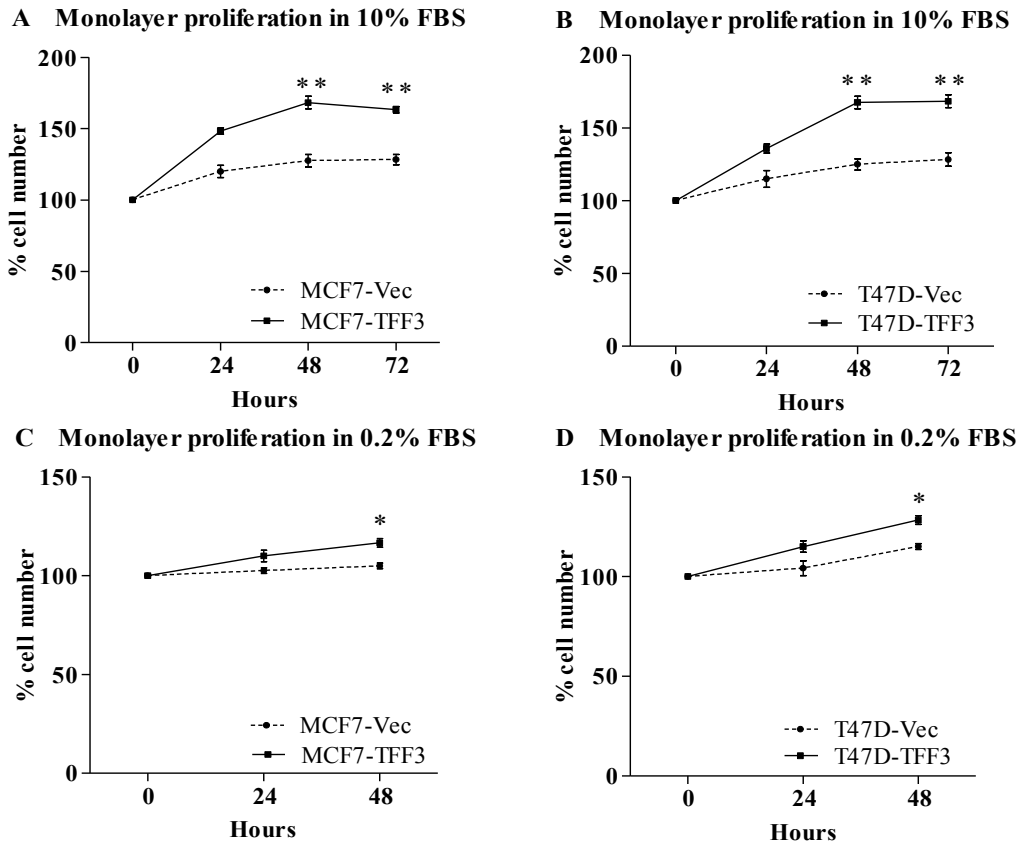


Figure 24: Forced expression of TFF3 in mammary carcinoma cells increased HUVEC monolayer proliferation. A, monolayer proliferation of HUVEC after co-culture with MCF-7 cells with forced expression of TFF3 in 10% FBS conditions. MCF-7 cells with empty vector were used as control. B, monolayer proliferation of HUVEC after co-culture with T47D cells with forced expression of TFF3 in 10% FBS conditions. T47D cells with empty vector were used as control. C, monolayer proliferation of HUVEC after co-culture with MCF-7 cells with forced expression of TFF3 in 0.2% FBS conditions. D, monolayer proliferation of HUVEC after co-culture with T47D cells with forced expression of TFF3 in 0.2% FBS conditions. *, $P < 0.05$; **, $P < 0.01$.

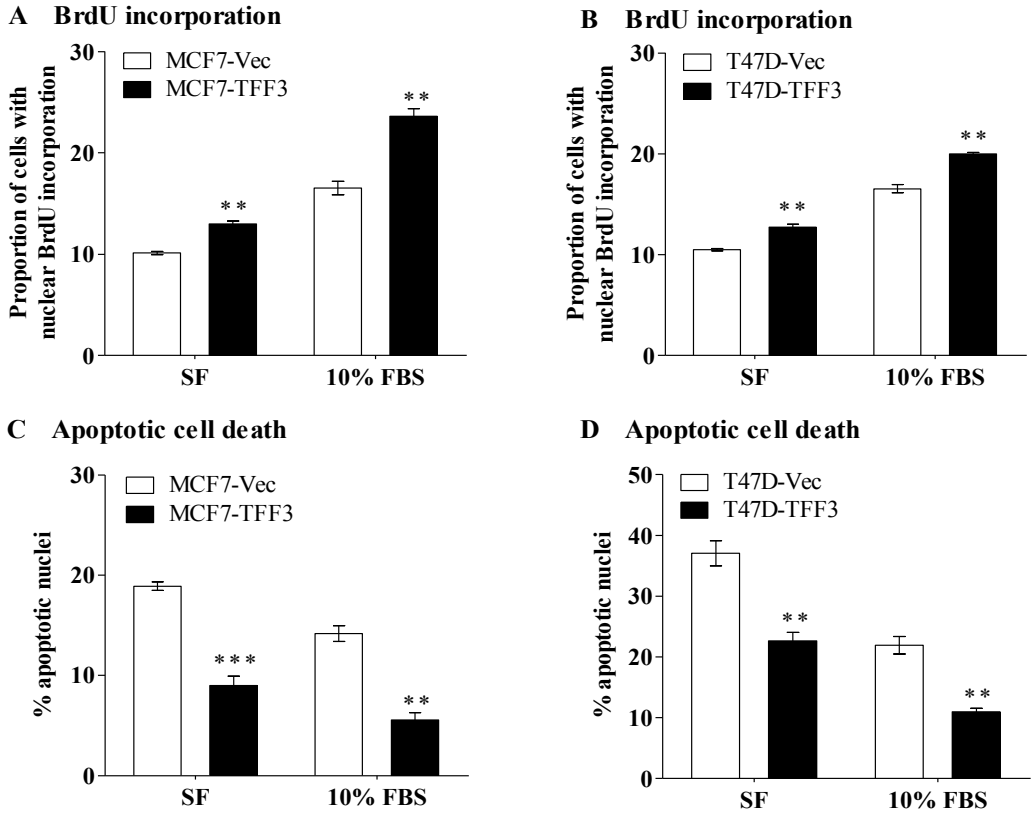


Figure 25: Forced expression of TFF3 in mammary carcinoma cells increased HUVEC cell cycle progression and survival. A, HUVEC cell cycle progression after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 in serum-free (SF) and 10% FBS conditions. MCF-7 cells with empty vector were used as control. B, HUVEC cell cycle progression after 24 hours co-culture with T47D with forced expression of TFF3 in serum-free (SF) and 10% FBS conditions. T47D cells with empty vector were used as control. C, HUVEC apoptotic cell death after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 in serum-free (SF) and 10% FBS conditions. D, HUVEC apoptotic cell death after 24 hours co-culture with T47D cells with forced expression of TFF3 in serum-free (SF) and 10% FBS conditions. **, $P < 0.01$; ***, $P < 0.001$.

3.2.4 Forced expression of TFF3 in mammary carcinoma cells promoted HUVEC migration, invasion, and tubule formation *in vitro*

During the process of *de novo* tumor angiogenesis, the surrounding tissue are invaded by endothelial cells to generate neovascularization the tumor (Lamalice *et al.*, 2007). I therefore determined the effects of TFF3 secreted from mammary carcinoma cells on the migratory and invasive behavior of HUVEC. Transwell migration and invasion assays were conducted in which HUVEC seeded on the membrane of transwell inserts (8.0 μm membrane

pore) were co-cultured with MCF-7 and T47D with forced expression of TFF3 plated in the bottom well of companion plate. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 significantly increased HUVEC migration by 121% and 100%, respectively as compared to the respective control cells (Figure 26A-B). Additionally, TFF3 secreted from MCF7-TFF3 and T47D-TFF3 significantly increased HUVEC invasion by 80% and 98% respectively as compared to the respective control cells (Figure 26C-D). TFF3 secreted from mammary carcinoma cells promoted HUVEC migration and invasion.

HUVEC retains the ability to form three-dimensional tubules in the Matrigel thereby stimulating angiogenesis *in vitro* (Bouïs *et al.*, 2001, Bagley *et al.*, 2003). The morphology of tubules formed by HUVEC in the Matrigel is representative of the capillary vessel formation *in vivo* (Donovan *et al.*, 2001, Bagley *et al.*, 2003). To determine if TFF3 secreted from mammary carcinoma cells stimulated angiogenic behavior of HUVEC, a tubule formation assay was conducted in which mammary carcinoma cells with forced expression of TFF3 were plated on the membrane of transwell insert co-cultured with HUVEC seeded in the Matrigel that coated in the bottom well of the companion plate. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 significantly increased tubule length and tubule number when compared with the respective control cells. TFF3 secreted from MCF7-TFF3 and T47D-TFF3 increased tubule length by 72% and 41%, respectively (Figure 27A-C) and tubule number by 28% and 30%, respectively (Figure 27B-D) when compared with the respective control cells. Co-culturing HUVEC with MCF7-TFF3 and T47D-TFF3 increased tubules formed by HUVEC in the Matrigel when compared with HUVEC co-cultured with the respective control cells (Figure 27E-F). Therefore, TFF3 secreted from mammary carcinoma cells promoted HUVEC tubule formation *in vitro*

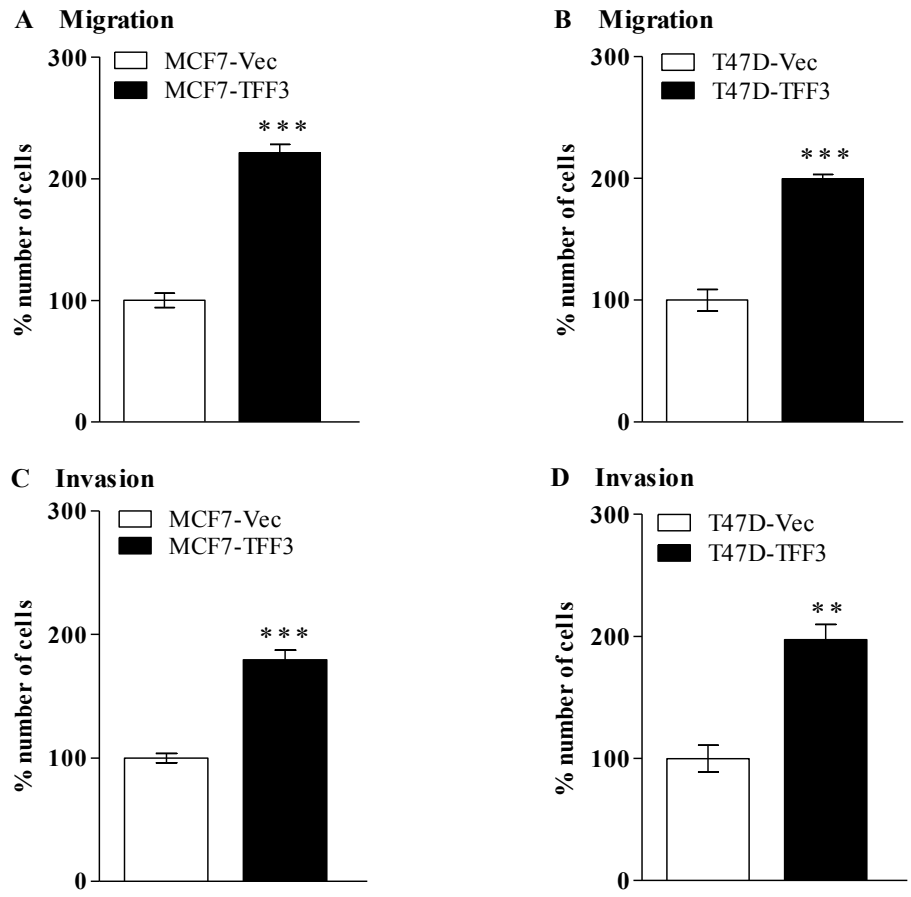


Figure 26: Forced expression of TFF3 in mammary carcinoma cells increased HUVEC migration and invasion. A, HUVEC migration after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 in serum-free conditions. MCF-7 cells with empty vector were used as control. B, HUVEC migration after 24 hours co-culture with T47D cells with forced expression of TFF3 in serum-free conditions. T47D cells with empty vector were used as control. C, HUVEC invasion after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 in serum-free conditions. D, HUVEC invasion after 24 hours co-culture with T47D cells with forced expression of TFF3 in serum-free conditions. **, $P < 0.01$; ***, $P < 0.001$.

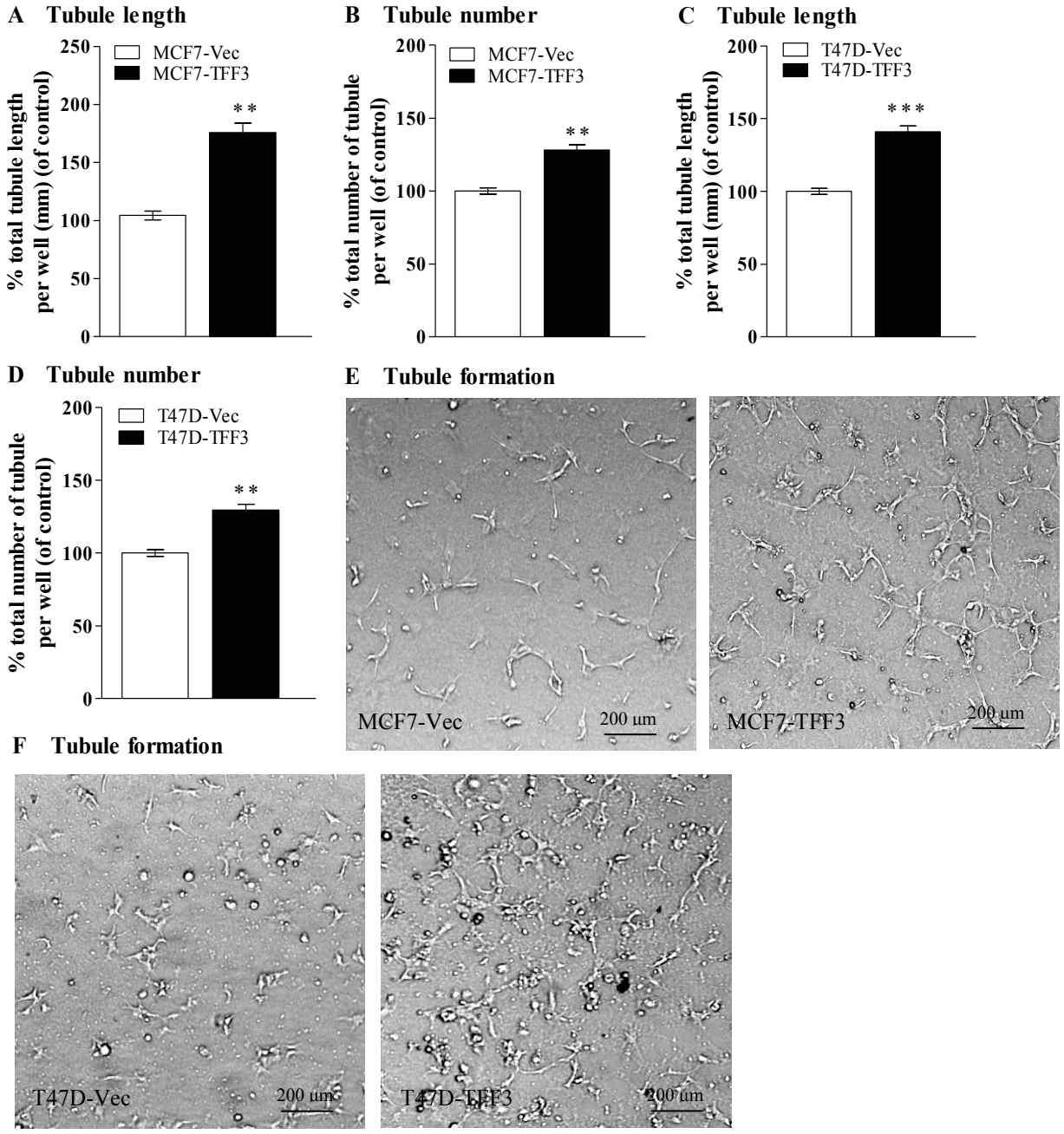


Figure 27: Forced expression of TFF3 in mammary carcinoma cells increased HUVEC tubule formation *in vitro*. A-B, HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with MCF-7 cells with forced expression of TFF3. MCF-7 cells with empty vector were used as control. Total tubule length (A) and total tubule number (B) were assessed. C and D, HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with T47D cells with forced expression of TFF3. T47D cells with empty vector were used as control. Total tubule length (C) and total tubule number (D) were assessed using ImageJ analysis software. E and F, representative light microscopy images of HUVEC tubule formation *in vitro* in the Matrigel after 12 hours co-culture with MCF-7 and T47D cells with forced expression of TFF3. The light microscopy images were taken at ×40 magnification. **, $P < 0.01$; ***, $P < 0.001$; scale bar, 200 µm.

3.2.5 Forced expression of TFF3 in mammary carcinoma cells promoted angiogenesis *in vivo*

To determine the potential *in vivo* role of TFF3 in *de novo* angiogenesis in mammary carcinoma, MCF7-Vec or MCF7-TFF3 cells were implanted into the mammary fat pad of immunodeficient nude mice and allowed to grow for 28 days. As previously reported, the tumors produced by MCF7-TFF3 were larger than those formed by MCF7-Vec After 4 weeks, indicating that TFF3 enhanced tumorigenicity *in vivo* (Kannan *et al.*, 2010). The proteins expression of IL-8, CD31, and CD34 in xenografts derived from MCF7-TFF3 and control MCF7-Vec were examined by IHC analysis. In xenograft models, MCF-7 cells with forced expression of TFF3 produced tumors with increased the expression of IL-8 protein as compared to tumors formed by control cells (Figure 28A), suggesting that TFF3 secreted from MCF-7 cells with forced expression of TFF3 promoted IL-8 protein expression. Tumor vasculature formed in xenografts was determined by analyzing microvessel density, which can be quantified by the area of CD31 and/or CD34 labeled cells. Endothelial cell surface marker, CD31 is an indicator of angiogenesis, cells expressing CD31 have higher angiogenic properties (Kim *et al.*, 2010). MCF-7 cells with forced expression of TFF3 produced tumors with increased area of CD31 labeled cells as compared to tumors formed by control cells (Figure 28A-B), indicating TFF3 secreted from mammary carcinoma cells significantly increased tumor microvessel density. CD34 (podoplanin) is a small transmembrane protein of lymphatic endothelium and molecular marker of lymphangiogenesis (Kahn and Marks, 2002). Lymphatic vasculature was determined by analyzing the area of CD34-labeled cells. IHC analysis demonstrated that a significantly increase area of CD34 labeled cells in tumors derived from MCF7-TFF3 when compared with tumors derived from control MCF7-Vec (Figure 28A-C), indicating that TFF3 secreted from mammary carcinoma cells increased lymphatic vascularization. These observations suggested that TFF3 secreted from mammary carcinoma cells promoted angiogenesis *in vivo.*

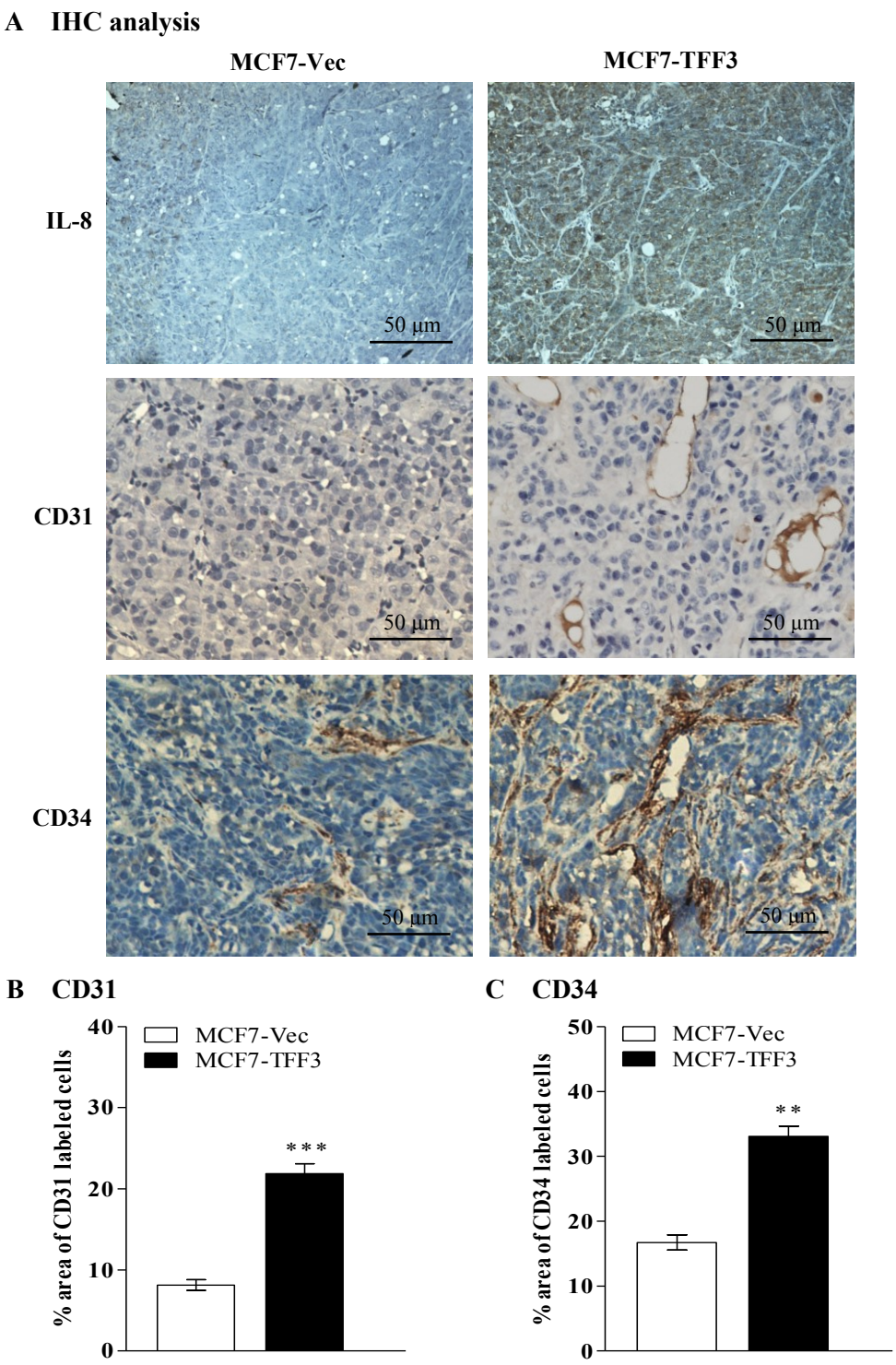


Figure 28: Forced expression of TFF3 in mammary carcinoma cells promoted angiogenesis *in vivo*. A, IHC analysis of IL-8, CD31, and CD34 proteins expression in xenograft tumors produced by control MCF7-Vec and MCF7-TFF3. B, Microvessel density (CD31) was assessed by quantifying percentage area of CD31 labeled cells in xenografts formed by control MCF7-Vec and MCF7-TFF3. C, CD34 was assessed by quantifying percentage area of CD34 labeled cells in xenografts formed by control MCF7-Vec and MCF7-TFF3. **, $P < 0.01$; ***, $P < 0.001$.

3.2.6 Depletion of TFF3 in mammary carcinoma cells by siRNA decreased TFF3 expression

The effects of depletion of endogenous TFF3 in mammary carcinoma cells on angiogenic behavior of HUVEC were also determined. MCF-7 and T47D cells were transiently transfected with a pSilencer 2.1-U6 hygro expression vector containing TFF3 siRNA (designated as MCF7-siTFF3 and T47D-siTFF3) or with a pSilencer 2.1-U6 hygro empty vector containing control siRNA (designated as MCF7-siVec and T47D-siVec) (Pandey *et al.*, 2014). The expression of TFF3 mRNA and protein in MCF-7 and T47D cells with depletion of TFF3 was verified by semi-quantitative RT-PCR and Western blot analysis. Depletion of TFF3 by siRNA in MCF-7 and T47D cells decreased TFF3 mRNA (Figure 29A-B) and decreased TFF3 protein (Figure 29C-D) when compared with the respective control cells. These data indicated that the endogenous expression of TFF3 in MCF-7 and T47D cells was depleted by siRNA.

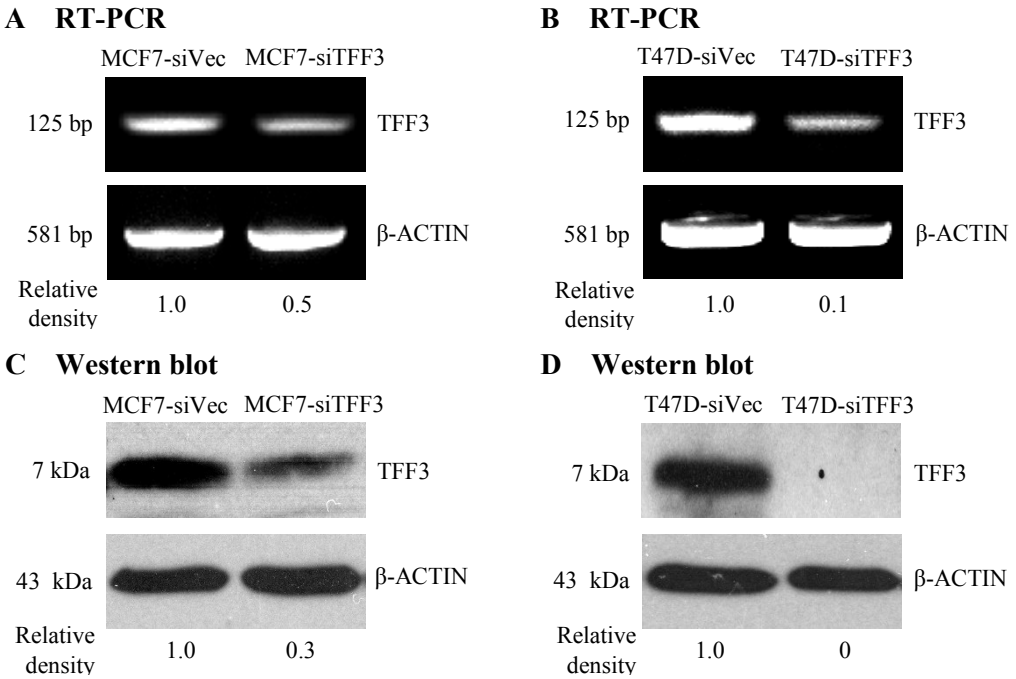


Figure 29: Depletion of TFF3 in mammary carcinoma cells decreased TFF3 expression. A, semi-quantitative RT-PCR analysis of the TFF3 mRNA expression in MCF-7 cells with depletion of TFF3. B, semi-quantitative RT-PCR analysis of the TFF3 mRNA expression in T47D cells with depletion of TFF3. C, Western blot analysis of TFF3 protein in MCF-7 cells with depletion of TFF3. D, Western blot analysis of TFF3 protein in T47D cells with depletion of TFF3. β-ACTIN was used as a loading control in both semi-quantitative RT-PCR and Western blot analysis.

3.2.7 Depletion of TFF3 in mammary carcinoma cells decreased HUVEC monolayer proliferation, cell cycle progression and survival

The effect of depletion of TFF3 in mammary carcinoma cells on HUVEC proliferation and survival were determined by co-culturing HUVEC with mammary carcinoma cells with depletion of TFF3. When compared with the respective control cells co-cultured with HUVEC, depletion of TFF3 in MCF-7 and T47D cells decreased HUVEC monolayer proliferation when compared with the respective control cells, in both 10% FBS conditions for 72 hours and 0.2% FBS conditions for 48 hours, respectively. Depletion of TFF3 by siRNA in MCF-7 and T47D cells significantly decreased HUVEC monolayer proliferation in 10% FBS conditions by 48% and 43%, respectively after 72 hours when compared with the respective control cells (Figure 30A-B). MCF-7 and T47D cells with depletion of TFF3 decreased HUVEC monolayer proliferation in 0.2% FBS condition by 18%

and 25%, respectively after 48 hours as compared with the respective control cells (Figure 30C-D). Depletion of TFF3 in mammary carcinoma cells decreased HUVEC monolayer cell proliferation.

Co-culturing HUVEC with MCF-7 and T47D cells with depletion of TFF3 significantly decreased HUVEC cell cycle progression, in both serum-free and 10% FBS conditions, when compared with HUVEC co-cultured with the respective control cells. Depletion of TFF3 by siRNA in MCF-7 and T47D cells significantly decreased HUVEC cell cycle progression in serum-free conditions by 27% and 30%, respectively as compared with the respective control cells (Figure 31A-B). MCF-7 and T47D cells with depletion of TFF3 significantly decreased HUVEC cell proliferation in 10% FBS conditions by 32% or 26%, respectively as compared with the respective control cells (Figure 31A-B). Furthermore, co-culture of HUVEC with MCF-7 and T47D cells with depletion of TFF3 significantly increased HUVEC apoptotic cell death in both serum-free and 10% FBS conditions, when compared with HUVEC co-cultured with the respective control cells. Depletion of TFF3 by siRNA in MCF-7 and T47D cells significantly increased HUVEC apoptotic cell death in serum-free conditions by 53% and 50%, respectively as compared with the respective control cells (Figure 31C-D). MCF-7 and T47D cells with depletion of TFF3 significantly increased HUVEC apoptotic cell death in 10% FBS conditions by 50% and 43%, respectively as compared with the respective control cells (Figure 31C-D). Depletion of TFF3 in mammary carcinoma cells by siRNA diminished HUVEC proliferation and survival.

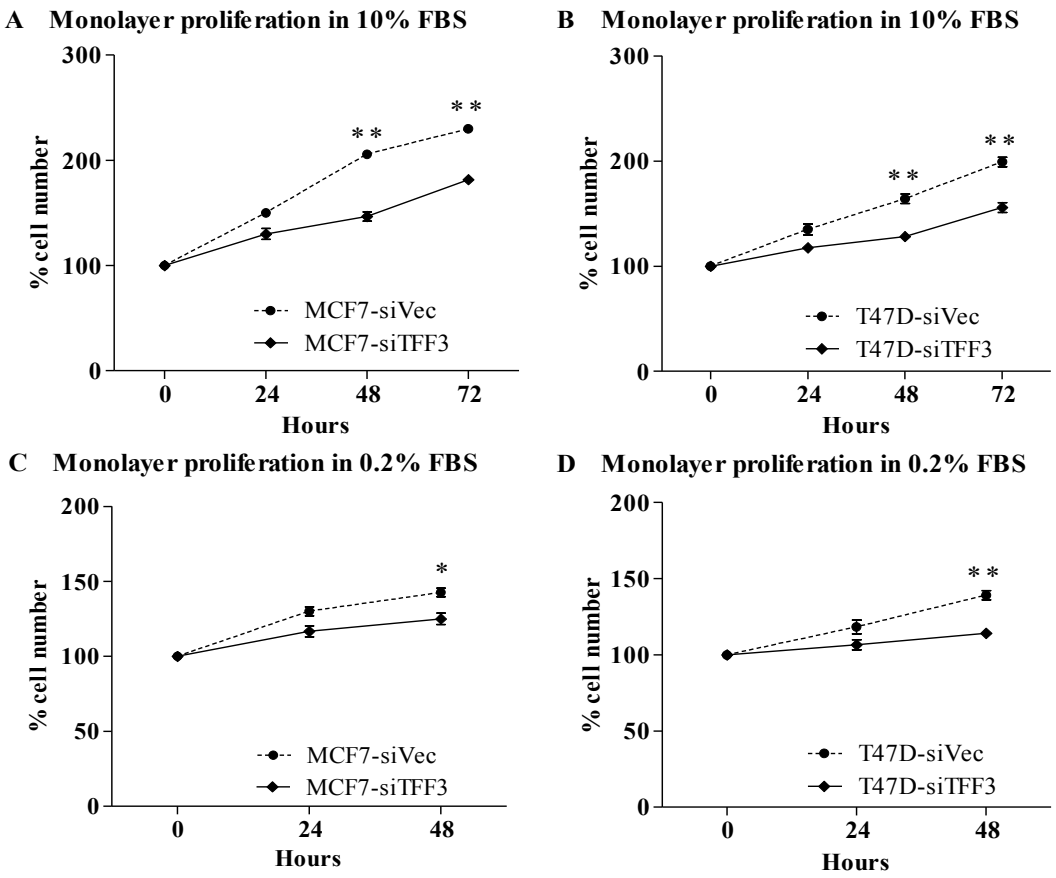


Figure 30: Depletion of TFF3 in mammary carcinoma cells decreased HUVEC monolayer proliferation. A, HUVEC monolayer proliferation after co-culture with MCF-7 cells with depletion of TFF3 in 10% FBS conditions. MCF-7 cells with siRNA control vector were used as control. B, HUVEC monolayer proliferation after co-culture with T47D cells with depletion of TFF3 in 10% FBS conditions. MCF-7 cells with siRNA control vector were used as control. C, HUVEC monolayer proliferation after co-culture with MCF-7 cells with depletion of TFF3 in 0.2% FBS conditions. D, HUVEC monolayer proliferation after co-culture with T47D cells with depletion of TFF3 in 0.2% FBS conditions.*, $P < 0.05$. **, $P < 0.01$.

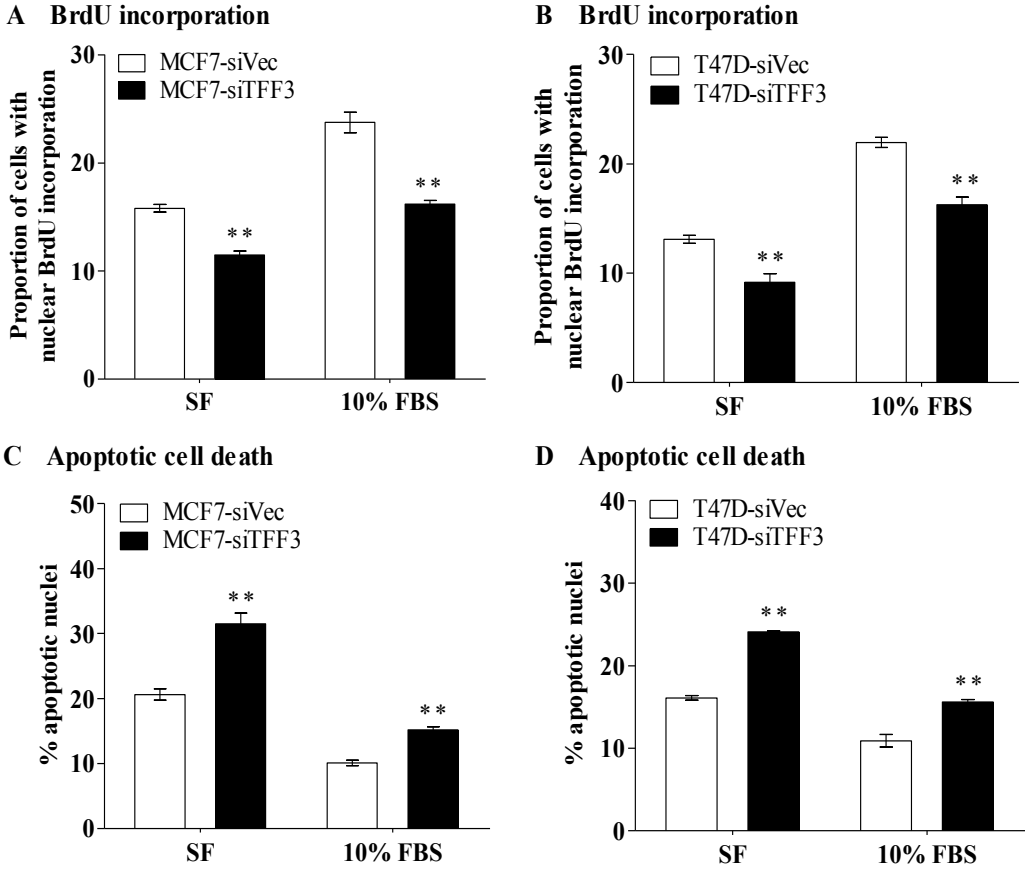


Figure 31: Depletion of TFF3 in mammary carcinoma cells decreased HUVEC cell cycle progression and survival. A, HUVEC cell cycle progression after 24 hours co-culture with MCF-7 cells with depletion of TFF3 in serum-free (SF) and 10% FBS conditions. MCF-7 cells with siRNA control vector were used as control. B, HUVEC cell cycle progression after 24 hours co-culture with T47D cells with depletion of TFF3 in serum-free (SF) and 10% FBS conditions. T47D cells with siRNA control vector were used as control. C, HUVEC apoptotic cell death after 24 hours co-culture with MCF-7 cells with depletion of TFF3 in serum-free (SF) and 10% FBS conditions. D, HUVEC apoptotic cell death after 24 hours co-culture with T47D cells with depletion of TFF3 in serum-free (SF) and 10% FBS conditions. **, $P < 0.01$.

3.2.8 Depletion of TFF3 in mammary carcinoma cells decreased HUVEC migration, invasion, and tube formation *in vitro*

The effect of depletion of TFF3 in mammary carcinoma cells on the migratory and invasive behavior of endothelial cells were determined by co-culturing HUVEC with mammary carcinoma cells with depletion of TFF3. Depletion of TFF3 in MCF-7 and T47D cells significantly decreased HUVEC migration by 41% and 45%, respectively (Figure 32A-B) and HUVEC invasion by 36% and 33%, respectively as compared with the respective control cells (Figure 32C-D). These data indicated that depletion of TFF3 diminished HUVEC migration and invasion.

To determine the effect of depletion of TFF3 by siRNA in mammary carcinoma cells on the angiogenic behaviors of HUVEC, a tubule formation assay was conducted by co-culturing depletion of TFF3 in MCF-7 and T47D cells with HUVEC seeded in the Matrigel. Depletion of TFF3 in MCF-7 and T47D cells by siRNA significantly decreased tubule length by 46% and 36%, respectively (Figure 33A-C) and tubule number by 38% and 30%, respectively (Figure 33B-D) when compared with the respective control cells. Depletion of TFF3 in MCF-7 and T47D cells decreased tubules formed by HUVEC in the Matrigel when compared with the respective control cells (Figure 33E-F). These data indicated that depletion of TFF3 inhibited HUVEC tubule formation *in vitro*. Depletion of TFF3 by siRNA in mammary carcinoma cells diminished angiogenic behaviors of HUVEC.

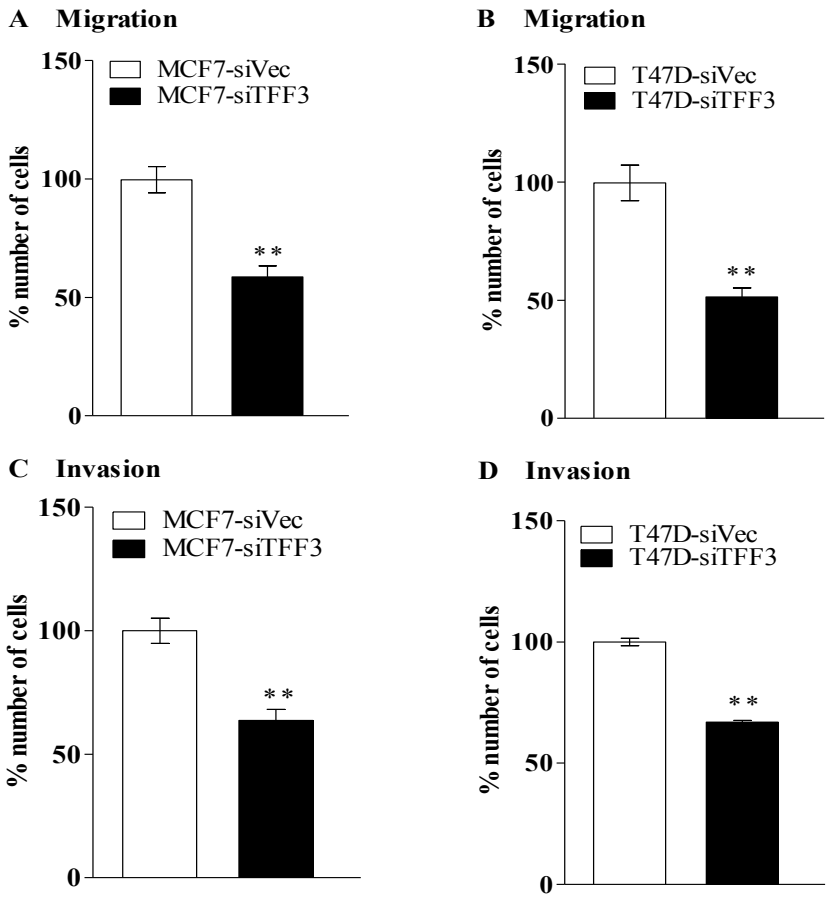


Figure 32: Depletion of TFF3 in mammary carcinoma cells decreased HUVEC migration and invasion. A, HUVEC migration after 24 hours co-culture with MCF-7 cells with depletion of TFF3. MCF-7 cells with siRNA control vector were used as control. B, HUVEC migration after 24 hours co-culture with T47D cells with depletion of TFF3. T47D cells with siRNA control vector were used as control. C, HUVEC invasion after 24 hours co-culture with MCF-7 cells with depletion of TFF3. D, HUVEC invasion after 24 hours co-culture with T47D cells with depletion of TFF3. **, $P < 0.01$.

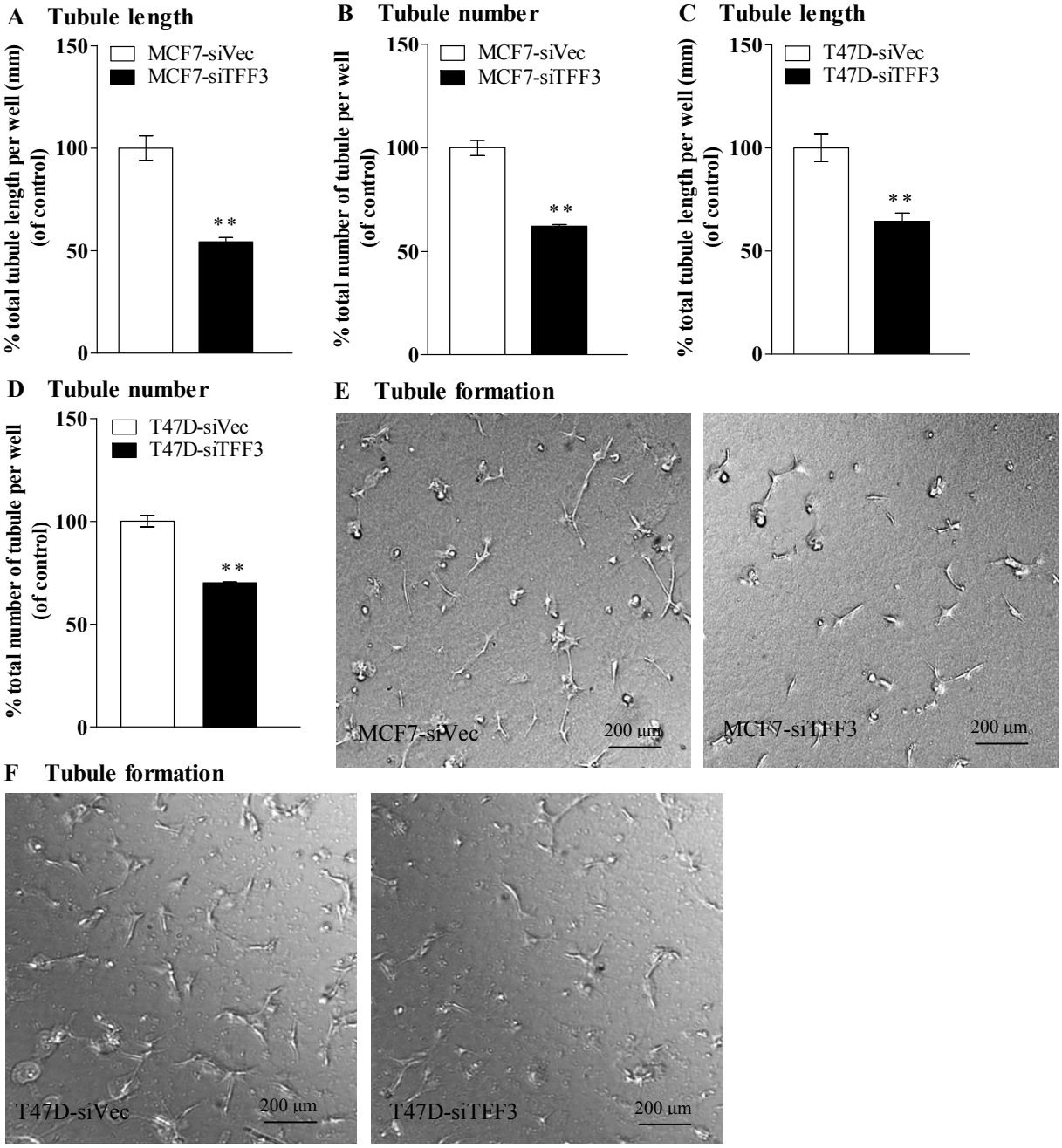


Figure 33: Depletion of TFF3 in mammary carcinoma cells decreased HUVEC tubule formation *in vitro*. A and B, HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with MCF-7 cell with depletion of TFF3 in serum-free conditions. MCF-7 cells with siRNA control vector were used as control. Total tubule length (A) and tubule number (B) were assessed after 12 hours incubation. C and D, HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with T47D cells with depletion of TFF3 in serum-free conditions. T47D cells with siRNA control vector were used as control. Total tubule length (C) and tubule number (D) were assessed after 12 hours incubation using ImageJ analysis software. E and F, representative light microscopy images of HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with MCF-7 and T47D cells with depletion of TFF3. The light microscopy images were taken at ×40 magnification. **, $P < 0.01$; scale bar, 200 µm.

3.2.9 Forced expression of TFF3 in mammary carcinoma cells increased IL-8 expression

According to the previously published microarray data set (Gene Expression Omnibus accession number GSE3494) (Miller *et al.*, 2005) as well as the gene expression profiling of MCF-7 with forced expression of TFF3 (Kannan *et al.*, 2010), several angiogenic markers associated with tumor angiogenesis were selected for further validation using real-time qPCR analysis. The qPCR analysis showed that forced expression of TFF3 in MCF-7 cells significantly increased the gene expression of VEGF-A (1.8-fold increase), IL-8 (5.5-fold increase) and transforming growth factor-beta (TGFB) (11.4-fold increase) as compared with the control MCF7-Vec (Figure 34). High level of TGF-beta is observed at the edges of breast tumor spread and is correlated with lymph node metastases (Dalal *et al.*, 1993). TGF-beta exhibits dual roles in promoting and suppression of tumor formation (Lebrun, 2012). It has previously reported that the motogenic activity of TFF3 for epithelial cells was not associated with alteration in the concentrations of TGF-beta protein and not affected by the presence of anti-TGF beta antibody (Dignass *et al.*, 1994). Hence, IL-8 was selected for further investigation the role of IL-8 in mammary carcinoma angiogenesis stimulated by TFF3. The expression of VEGF-A and IL-8 in mammary carcinoma cells with forced expression of TFF3 was analyzed by semi-quantitative RT-PCR and Western blot or ELISA analysis. The qPCR analysis showed that the VEGF-A gene expression was slightly up-regulated by TFF3, but the expression of VEGF-A mRNA in MCF-7 cells with forced expression of TFF3 appeared to be not markedly different from the control MCF7-Vec (Figure 35A). This discrepancy probably due to the semi-quantitative RT-PCR was not able to detect the relative differences. The protein expression of VEGF-A in MCF-7 cells with forced expression of TFF3 was relatively similar to the control MCF7-Vec (Figure 35B). This result indicated that the expression of VEGF-A was probably not regulated by TFF3. Mammary carcinoma cells endogenously expressed relatively low level of IL-8 mRNA (Figure 35C), but forced expression of TFF3 in MCF-7 and T47D cells increased IL-8 mRNA

level (Figure 35D-E) and increased IL-8 protein secreted to the medium (Figure 35F-G) as compared with the respective control cells. These data suggested that TFF3 stimulated the expression of IL-8 in mammary carcinoma cells.

The functional receptors for IL-8, CXCR1 and CXCR2, are present in different cells including tumors and endothelial cells, and have been demonstrated to involve in tumor angiogenesis (Richards *et al.*, 1997, Kitadai *et al.*, 1998, Miller *et al.*, 1998). Besides over-expression of IL-8, the differential expression of IL-8 receptors in benign and malignant breast tissues has been reported (Miller *et al.*, 1998). The expression of CXCR1 and CXCR2 mRNAs were observed in the mammary carcinoma cells with forced expression of TFF3 (Figure 36A-B).

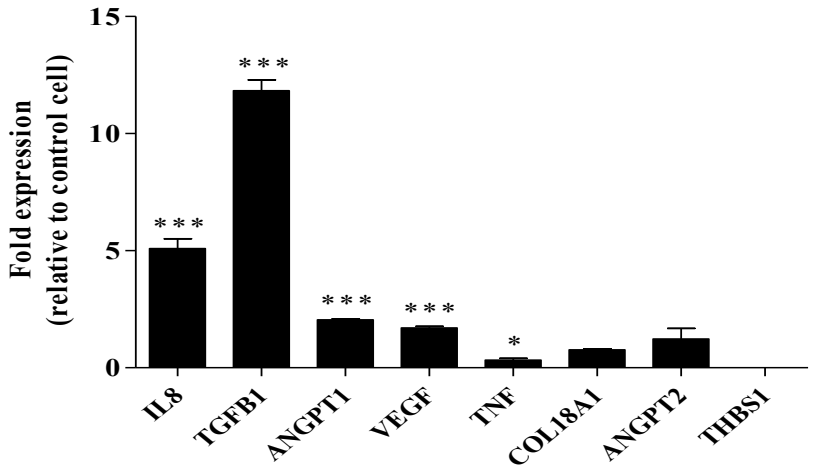


Figure 34: Real-time qPCR analysis of the gene expression of angiogenic markers modulated by TFF3. Fold expression indicated gene expression of angiogenic markers in MCF-7 cells with forced expression of TFF3 relative to control MCF7-Vec. Fold expression ≥ 2 indicated the gene expression of angiogenic markers was up-regulated by TFF3. *, $P < 0.05$; ***, $P < 0.001$. Pro-angiogenic markers included IL-8, interleukin 8; TGFB, Transforming growth factor beta; ANGPT1, Angiopoietin 1; VEGF-A, Vascular endothelial growth factor; TNF; Tumor necrosis factor and anti-angiogenic markers included COL18A1, Collagen alpha-1(XVIII) chain; ANGPT2, Angiopoietin 2; THBS1, Thrombospondin 1.

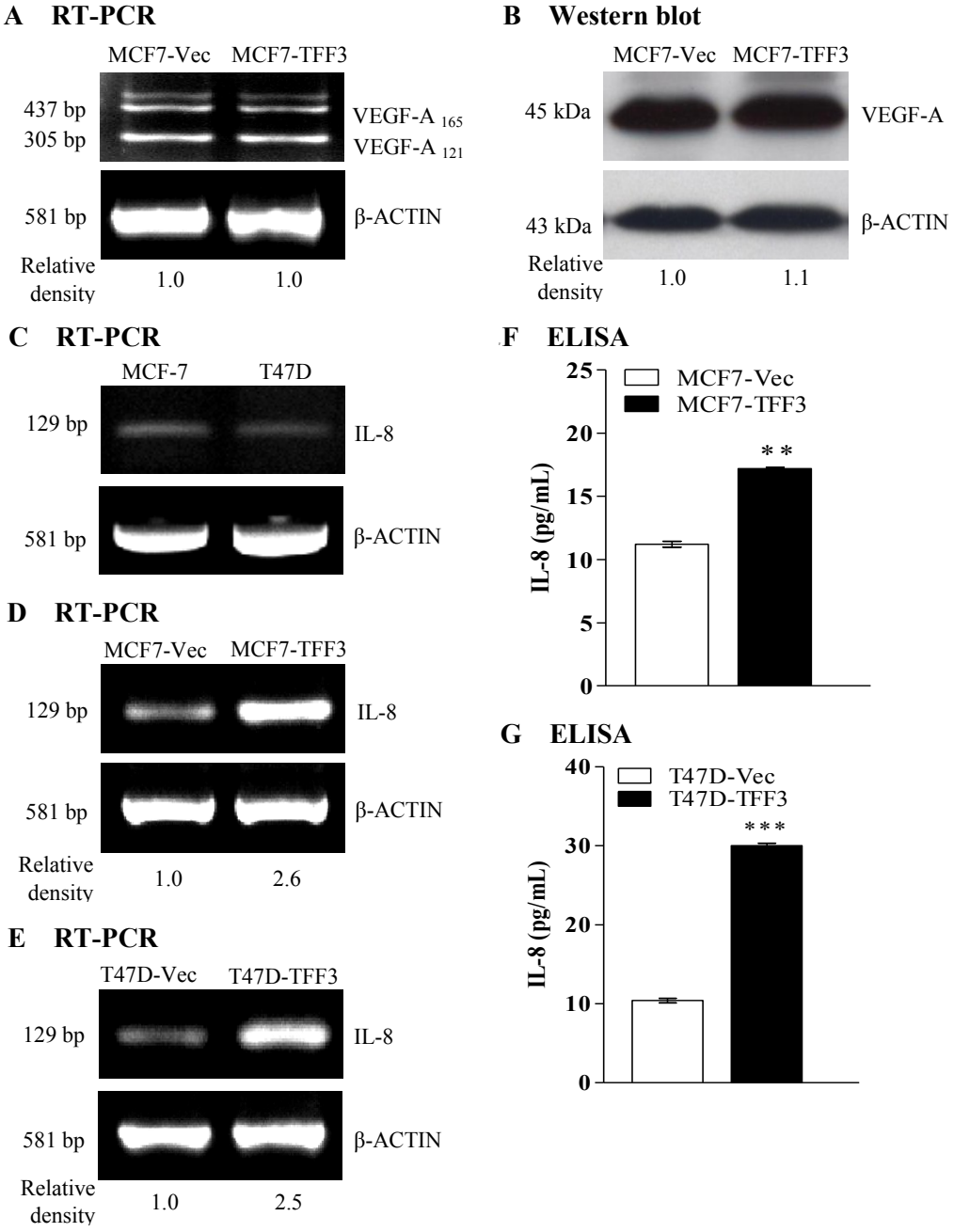


Figure 35: Forced expression of TFF3 in mammary carcinoma cells increased IL-8 expression. A, semi-quantitative RT-PCR analysis of VEGF-A mRNA level in MCF-7 cells with forced expression of TFF3. MCF-7 cells with empty vector were used as control. B, Western blot analysis of VEGF-A protein in MCF-7 cells with forced expression of TFF3. C, semi-quantitative RT-PCR analysis of endogenous expression of IL-8 mRNA in MCF-7 and T47D cells. D, semi-quantitative RT-PCR analysis of the expression of IL-8 mRNA in MCF-7 with forced expression of TFF3. E, semi-quantitative RT-PCR analysis of the expression of IL-8 mRNA in T47D cells with forced expression of TFF3. T47D cells with empty vector were used as control. F, ELISA analysis of IL-8 protein secreted to the medium by MCF-7 cells with forced expression of TFF3. G, ELISA analysis of IL-8 protein secreted to the medium by T47D cells with forced expression of TFF3. β-ACTIN was used as a loading control in both semi-quantitative RT-PCR and Western blot. **, $P < 0.01$, ***, $P < 0.001$.

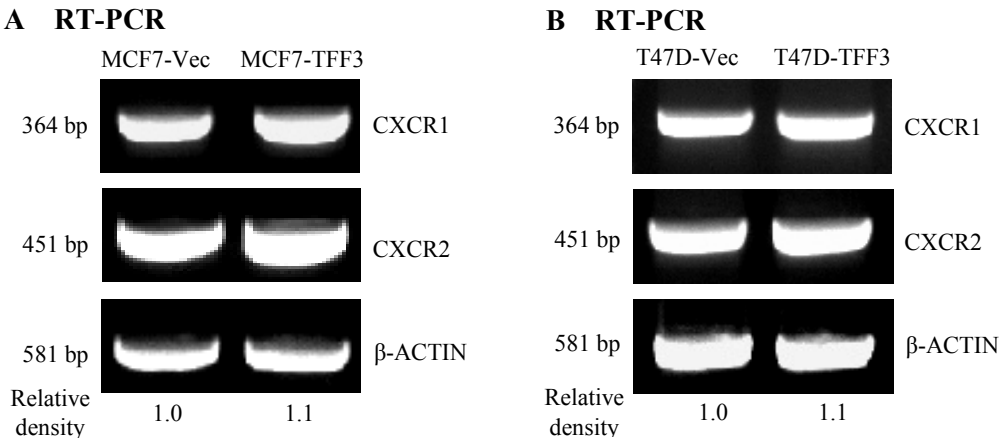


Figure 36: Expression of CXCR1 and CXCR2 mRNA in mammary carcinoma cells with forced expression of TFF3. A, semi-quantitative RT-PCR analysis of the expression of CXCR1 and CXCR2 mRNA in MCF-7 cells with forced expression of TFF3. MCF-7 cells with empty vector were used as control. B, semi-quantitative RT-PCR analysis of the expression of CXCR1 and CXCR2 mRNA in T47D cells with forced expression of TFF3. T47D cells with empty vector were used as control. β-ACTIN was used as a loading control in semi-quantitative RT-PCR.

3.2.10 Forced expression of TFF3 in mammary carcinoma cells increased IL-8 promoter activity

IL-8 protein is secreted in normal tissue to maintain homeostasis, but its expression is rapidly increased in response to pro-inflammatory cytokines or growth factors. The production of IL-8 is regulated by the combinatorial activation of transcription factors and their interaction with the IL-8 promoter gene (Lakshminarayanan *et al.*, 1998, Roebuck *et al.*, 1999). It is known that the proximal region of IL-8 promoter contains functional binding sites for the transcription factors namely NF-IL-6 (-94 to -81 bp), AP-1 (-126 to -120 bp) and NF-κB (-80 to -70 bp) (Mukaida *et al.*, 1994). Besides these binding sites, additional potential binding sites for STAT3 (-245 to -237 bp) have also been identified (Oka *et al.*, 2010). Firstly, I determined if TFF3 stimulates IL-8 promoter activity, MCF-7 and T47D cells with forced expression of TFF3 were transiently transfected with a full length of IL-8 promoter reporter vector (full length, -4800 to + 104 bp) and a pRL-CMV control reporter vector. MCF-7 and T47D cells with forced expression of TFF3 significantly increased IL-8 promoter activity by 4.5-fold and 2.2-fold respectively when compared with the respective control cells (Figure

37A-B), indicating that TFF3 stimulated IL-8 promoter activity in mammary carcinoma cells. The cooperative actions of NF-κB, AP-1, and NF-IL-6 transcription factors increase the expression of IL-8 protein (Mukaida *et al.*, 1990, Mukaida *et al.*, 1994, Roebuck, 1999). Secondly, I determined if these transcription factors are involved in enhancing IL-8 promoter activity in mammary carcinoma cells stimulated by TFF3, MCF-7 and T47D with forced expression of TFF3 were transiently transfected with a shorter IL-8 promoter, pGL-IL8-152 reporter vector (-152 to +44 bp) containing transcription factors namely NF-κB, AP-1, and NF-IL-6 , and a pRL-CMV control reporter vector. MCF-7 and T47D cells with forced expression of TFF3 increased IL-8 promoter activity when compared with the respective control MCF7-Vec and T47D-Vec transiently transfected with pGL-IL8-152 reporter vector (Figure 37C-D). The binding sites for AP-1, NF-IL-6 and NF-κB, present at the proximal region of IL-8 promoter, were also involved in enhancing IL-8 promoter activity stimulated by TFF3 in mammary carcinoma cells.

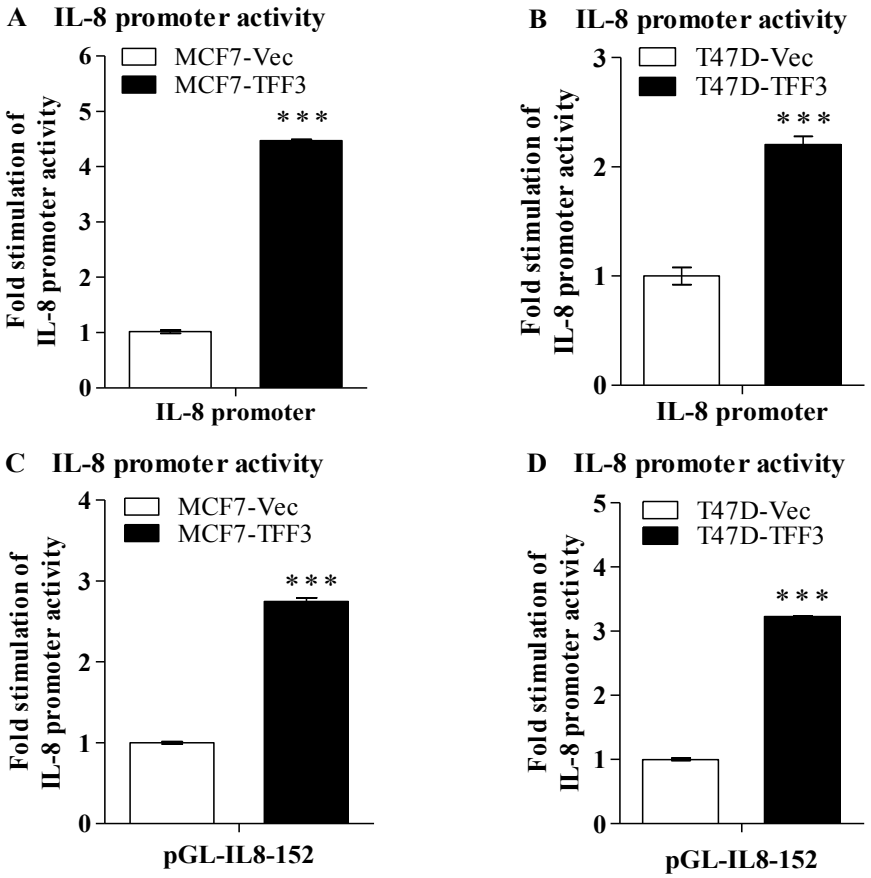


Figure 37: Forced expression of TFF3 in mammary carcinoma cells increased IL-8 promoter activity. A, IL-8 promoter activity (full length, -4800 to + 104 bp) in MCF-7 cells with forced expression of TFF3. MCF-7 cells with empty vector were used as control. B, IL-8 promoter activity in T47D cells with forced expression of TFF3. T47D cells with empty vector were used as control. C, IL-8 promoter activity in MCF7-Vec and MCF7-TFF3 transiently transfected with pGL-IL8-152 reporter (-152 to +44 bp). D, IL-8 promoter activity in T47D-Vec and T47D-TFF3 transiently transfected pGL-IL8-152 reporter. Reporter constructs consist of 5' flanking regions of the IL-8 gene were subcloned upstream of a luciferase reporter gene in the pGL3 basic vector. pGL-IL8-152 reporter construct encompassing nucleotides -152 to +44 of the IL-8 promoter, which was consist of NF-IL6, AP-1, NFκB binding sites. The TATA box, and the NF-IL6, NFκB and AP-1 binding sites are located at -13, -70, -80 and -120, respectively. Fold stimulation was measured by the ratio firefly luciferase of IL-8 reporter in MCF7-TFF3 and T47D-TFF3 to firefly luciferase of IL-8 reporter in MCF7-Vec and T47D-Vec. ***, $P < 0.001$.

3.2.11 Depletion of TFF3 in mammary carcinoma cells decreased IL-8 promoter activity

To determine if depletion of TFF3 by siRNA in mammary carcinoma cells decreased IL-8 expression, mammary carcinoma cells with depletion of TFF3 were transiently transfected with an IL-8 reporter vectors including IL-8 promoter (-1480 to +104 bp) or pGL-IL8-152 reporter vector, together with a pRL-CMV control reporter vector. MCF-7 and T47D cells with depletion of TFF3 and transiently transfected with a full length of IL-8 promoter (-1480 to +104 bp) exhibited significantly decreased IL-8 promoter activity by 3.4-fold or 1.7-fold respectively when compared with the respective control cells (Figure 38A-B). Additionally, MCF-7 and T47D cells with depletion of TFF3 and transiently transfected with a shorter IL-8 promoter, pGL-IL8-152 reporter vector, exhibited significantly decreased IL-8 promoter activity when compared with the respective control cells (Figure 38C-D). These data indicated that depletion of TFF3 in mammary carcinoma cells by siRNA abrogated IL-8 promoter activity and the transcription factors namely AP-1, NF-IL-6 and NF-κB were necessary for TFF3-stimulated IL-8 promoter activity. Subsequently, depletion of TFF3 in MCF-7 and T47D cells decreased IL-8 mRNA (Figure 39A-B) and IL-8 protein secreted to the medium (Figure 39C-D) when compared with the respective control cells. Depletion of TFF3 by siRNA reduced IL-8 promoter activity and subsequently decreased the expression of IL-8 in mammary carcinoma cells.

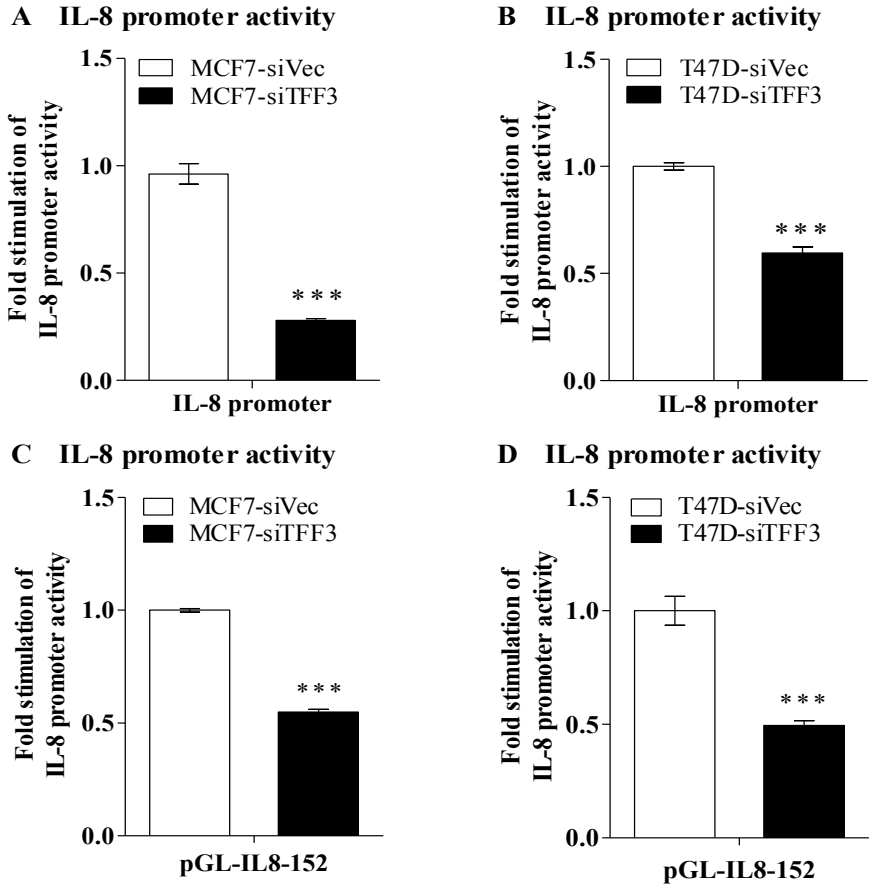


Figure 38: Depletion of TFF3 in mammary carcinoma cells decreased IL-8 promoter activity. A, IL-8 promoter reporter activity (full length, -4800 to + 104 bp) in MCF-7 cells with depletion of TFF3. MCF-7 cells with empty vector were used as control. B, IL-8 promoter reporter activity (full length, -4800 to + 104 bp) in T47D cells with depletion of TFF3. T47D cells with empty vector were used as control. C, IL-8 promoter activity in MCF7-Vec and MCF7-TFF3 transiently transfected with a shorter IL-8 promoter namely pGL-IL8-152 reporter. D, IL-8 promoter activity in T47D-Vec and T47D-TFF3 transiently transfected pGL-IL8-152 reporter. . ***, $P < 0.001$.

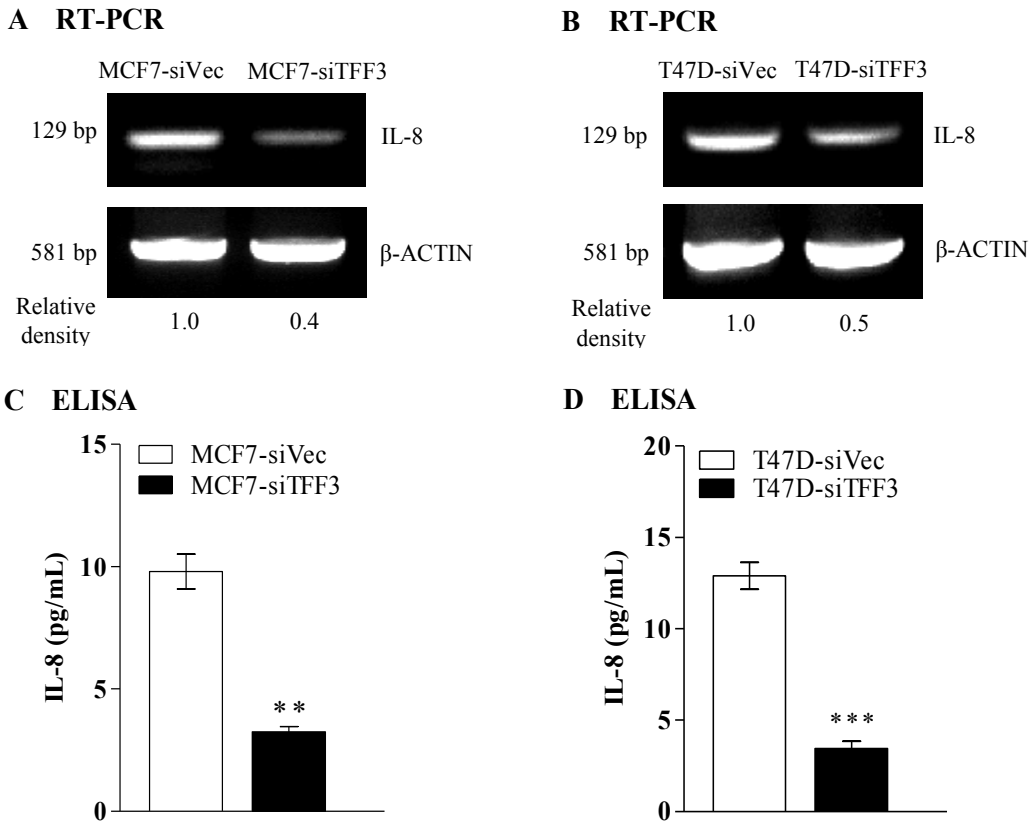


Figure 39: Depletion of TFF3 in mammary carcinoma cells decreased IL-8 expression. A, semi-quantitative RT-PCR analysis of IL-8 mRNA in MCF-7 cells with depletion of TFF3. MCF-7 cells with siRNA control vector were used as control. B, semi-quantitative RT-PCR analysis of IL-8 mRNA in T47D cells depletion of TFF3. T47D cells with siRNA control vector were used as control. C, ELISA analysis of IL-8 protein secreted to the medium by MCF-7 cells with depletion of TFF3. D, ELISA analysis of IL-8 protein secreted to the medium by T47D cells with depletion of TFF3. β-ACTIN was used as a loading control in semi-quantitative RT-PCR. **, $P < 0.01$, ***, $P < 0.001$.

3.2.12 Depletion of IL-8 by siRNA abrogated the stimulatory effect of TFF3 on HUVEC migration, invasion, and tubule formation *in vitro*

To determine if IL-8 mediated the stimulatory effect of TFF3 on the angiogenic behaviors of HUVEC, an IL-8 siRNA was employed to deplete the expression of IL-8 in MCF-7 cells with forced expression of TFF3. Hence, MCF-7 cells with forced expression of TFF3 were transiently transfected with an IL-8 siRNA (designated as siIL-8) or a scrambled control siRNA (designated as siControl). MCF-7 cells with forced expression of TFF3 and transiently transfected with IL-8 siRNA appeared to relatively decrease the expression of IL-8 mRNA (after 24 hours transfection) (Figure 40A). Semi-quantitative RT-PCR was not sensitive enough to detect the relative discrepancy of the IL-8 mRNA expression between MCF-7 cells with forced expression of TFF3 transiently transfected with IL-8 siRNA and those transiently transfected with scrambled control siRNA. Therefore, ELISA was used to quantify the concentration of IL-8 protein secreted to the medium. MCF-7 cells with forced expression of TFF3 and transiently transfected with IL-8 siRNA decreased IL-8 protein secreted to the medium (after 48 hours and 72 hours transfection) (Figure 40B-C) when compared with the respective siControl cells. IL-8 siRNA selectively depleted the expression of IL-8 in MCF-7 cells with forced expression of TFF3 and vector control cells. Subsequently, HUVEC were co-cultured with MCF7-Vec or MCF7-TFF3 with depletion of IL-8. Depletion of IL-8 in MCF7-Vec significantly decreased HUVEC migration by 42% (Figure 41A) and invasion by 42% (Figure 41B) when compared with the MCF7-Vec transiently transfected with control siRNA. Depletion of IL-8 in MCF7-TFF3 significantly decreased HUVEC migration by 74% (Figure 41A) and invasion by 65% (Figure 41B) when compared with MCF7-TFF3 transiently transfected with control siRNA, indicating that the depletion of IL-8 by siRNA abrogated the stimulatory effect of TFF3 on HUVEC migration and invasion. Furthermore, MCF7-Vec with depletion of IL-8 by siRNA significantly decreased tubule length by 43% (Figure 41C) and tubule number by 37% (Figure 41D) when compared with MCF7-Vec transiently transfected with control siRNA, indicating that depletion of IL-8 by

siRNA inhibited angiogenic effect of IL-8 on HUVEC tubule formation *in vitro*. Depletion of IL-8 in MCF7-TFF3 significantly decreased tubule length by 61% (Figure 41C) and tubule number by 58% (Figure 41D) when compared with MCF7-TFF3 transiently transfected with control siRNA, indicating that depletion of IL-8 by siRNA abrogated the stimulatory effects of TFF3 on HUVEC tubule formation *in vitro*. Depletion of IL-8 by siRNA in MCF-7 cells with forced expression of TFF3 significantly decreased tubules formed by the HUVEC in the Matrigel when compared with the control cells (Figure 41E). These data indicated that depletion of IL-8 by siRNA decreased the stimulatory effects of TFF3 on HUVEC tubule formation *in vitro*. Depletion of IL-8 in MCF-7 cells by siRNA abrogated the ability of TFF3 to stimulate angiogenic behaviors of HUVEC.

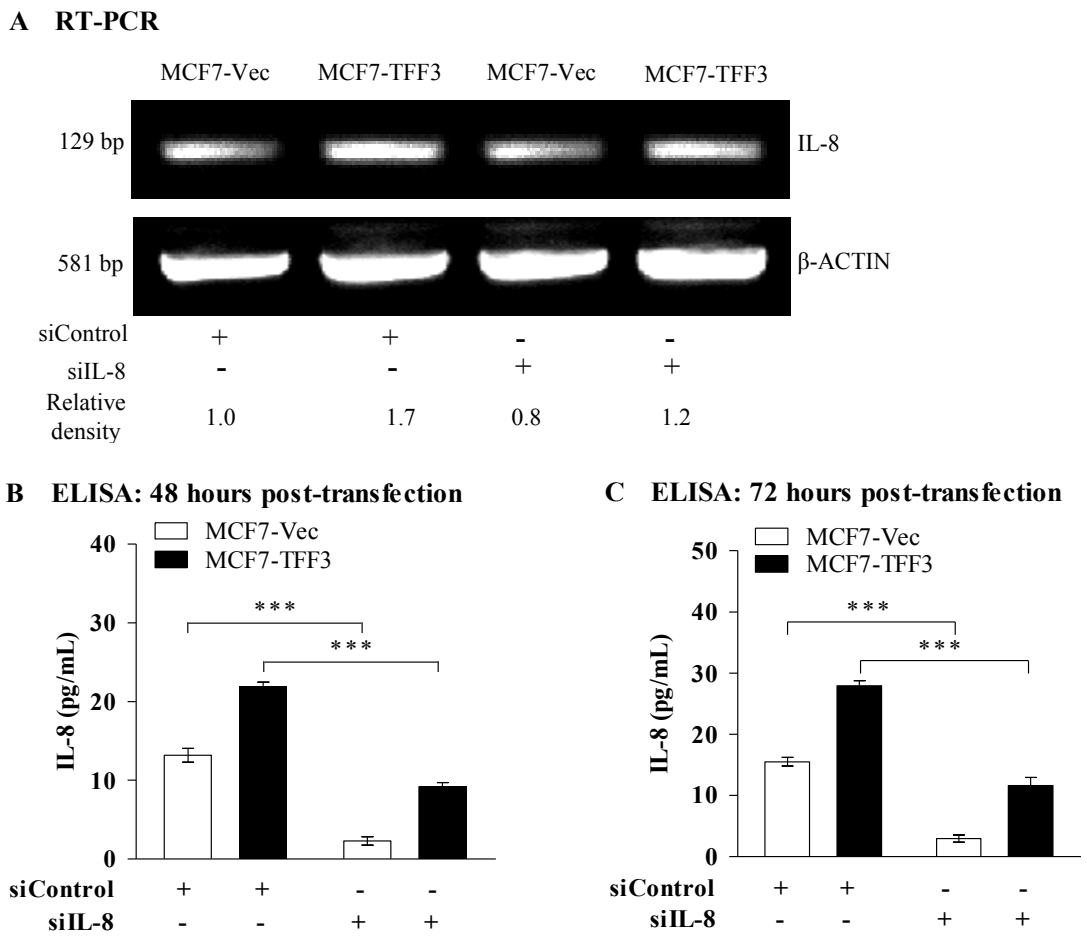
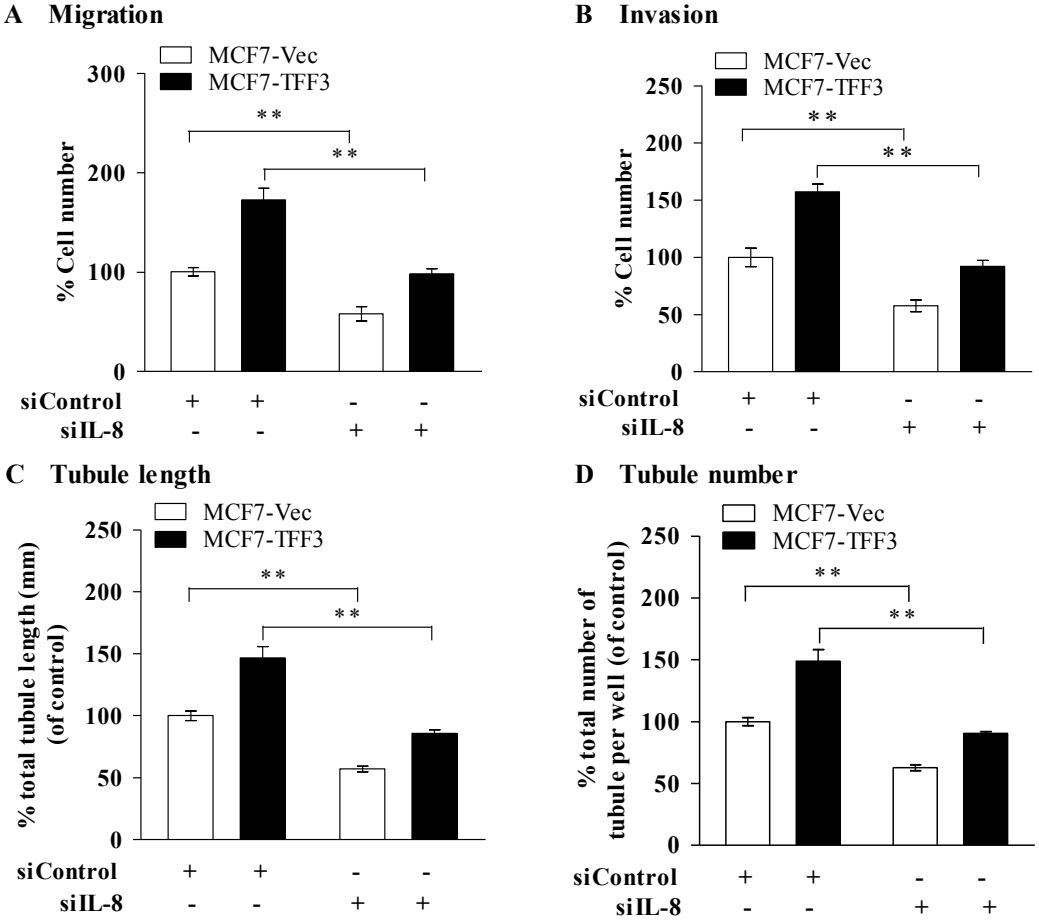


Figure 40: Depletion of IL-8 in mammary carcinoma cells with forced expression of TFF3 by siRNA decreased IL-8 expression. A, semi-quantitative RT-PCR of the expression of IL-8 mRNA in MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or IL-8 siRNA after 24 hours transfection. Scrambled control siRNA was used as control. MCF7-Vec transiently transfected with scrambled control siRNA was used as baseline. B, ELISA analysis of IL-8 protein secreted to the medium by MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or IL-8 siRNA after 48 hours transfection. C, ELISA analysis of IL-8 protein secreted to the medium by MCF-7 with forced expression of TFF3 transiently transfected with control siRNA or IL-8 siRNA after 72 hours transfection. β- ACTIN was used as a loading control in semi-quantitative RT-PCR. ***, $P < 0.001$ as compared with MCF7-Vec and MCF7-TFF3 transiently transfected with control siRNA.



E Tubule formation

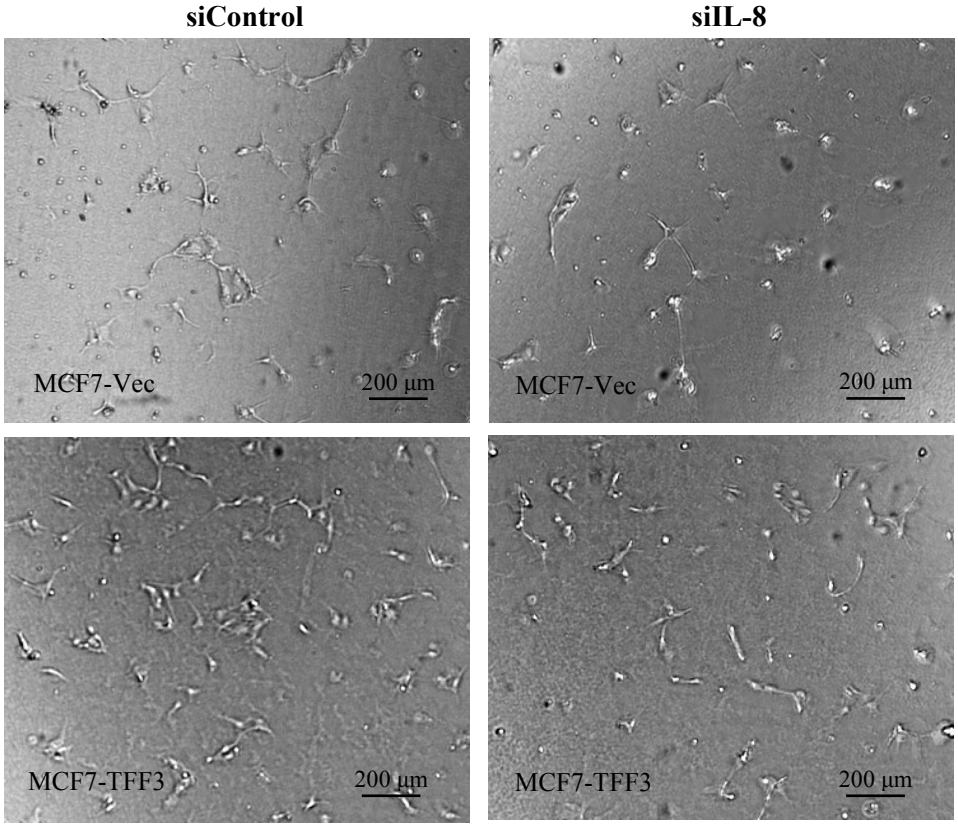


Figure 41: Depletion of IL-8 in mammary carcinoma cells by siRNA abrogated stimulatory effect of TFF3 on HUVEC migration, invasion, and tubule formation *in vitro*. A, HUVEC migration after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or IL-8 siRNA in serum-free conditions. Scrambled control siRNA was used as control. MCF7-Vec transiently transfected with scrambled control siRNA was used as baseline. B, HUVEC invasion after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or IL-8 siRNA in serum-free conditions. C and D, HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or IL-8 siRNA in serum-free conditions for 12 hours incubation. Total tubule length (C) and tubule number (D) was assessed using ImageJ analysis software. E, representative light microscopy images of HUVEC tubule formation *in vitro* in the Matrigel after 12 hours co-culture of with MCF7-Vec and MCF7-TFF3 transiently transfected with control siRNA or IL-8 siRNA. The light microscopy images were taken at ×40 magnification. **, $P < 0.01$ as compared with MCF7-Vec and MCF7-TFF3 transiently transfected with control siRNA; scale bar, 200 μm.

3.2.13 Blocking IL-8 in mammary carcinoma cells by anti-IL-8 monoclonal antibody inhibited the stimulatory effect of TFF3 on HUVEC tubule formation *in vitro*

The development of humanized monoclonal antibodies against IL-8 has been exploited to inhibit the tumor-promoting effect of IL-8 in melanoma and bladder xenograft models (Huang *et al.*, 2002, Mian *et al.*, 2003). Humanized anti-IL-8 monoclonal antibodies (e.g., ABX-IL8) bind to IL-8 and prevent it from binding to its receptors (Yang *et al.*, 1999, Huang *et al.*, 2002, Mian *et al.*, 2003). Administration of anti-IL-8 antibodies suppress IL-8 signaling on tumor progression and development (Huang *et al.*, 2002). Accordingly, an anti-IL-8 monoclonal antibody was utilized for blocking IL-8 in MCF-7 cells with forced expression of TFF3 and vector control cells. To initially determine the effect of blocking IL-8 in control MCF7-Vec on the IL-8-mediated HUVEC tubule formation *in vitro*, HUVEC were co-cultured with MCF7-Vec treated with different concentration of anti-IL-8 monoclonal antibody (2.5, 5.0, 10.0, 20.0, 50.0 μg/mL) or IgG control (50.0 μg/mL) . Blocking IL-8 in MCF7-Vec by different concentration of anti-IL-8 monoclonal antibody decreased tubule length in a concentration-dependent manner, with 57% reduction of HUVEC tubule formation *in vitro* at 50 μg/mL anti-IL-8 monoclonal antibody (Figure 42A). These data indicated that

blocking of IL-8 in control MCF7-Vec by anti-IL-8 monoclonal antibody decreased HUVEC tubule formation *in vitro*.

To determine if blocking of IL-8 by anti-IL-8 monoclonal antibody inhibited the stimulatory effect of TFF3 on HUVEC tubule formation *in vitro*, HUVEC were co-cultured with MCF7-Vec and MCF7-TFF3 treated with 50 μg/mL of anti-IL-8 monoclonal antibody or IgG control. Blocking of IL-8 in MCF7-Vec by anti-IL-8 monoclonal antibody significantly decreased tubule length by 39% (Figure 42B) and tubule number by 40% (Figure 42C) when compared with MCF7-Vec treated with IgG control, indicating that inhibition of IL-8 in MCF7-Vec by anti-IL-8 monoclonal antibody attenuated IL-8-mediated angiogenesis *in vitro*. Blocking of IL-8 in MCF7-TFF3 by anti-IL-8 monoclonal antibody significantly decreased tubule length by 52% (Figure 42B) and tubule number by 49% (Figure 42C) when compared with MCF7-TFF3 treated with IgG control, indicating that blocking of IL-8 in MCF7-TFF3 cells by anti-IL-8 monoclonal antibody inhibited the stimulatory effect of TFF3 in angiogenesis *in vitro*. Blocking of IL-8 in MCF7-Vec or MCF7-TFF3 by anti-IL-8 monoclonal antibody significantly decreased tubules formed by HUVEC in the Matrigel when compared with the respective cells treated with IgG control (Figure 42D). Depletion of IL-8 in MCF-7 cells by anti-IL-8 monoclonal antibody inhibited the ability of TFF3 to stimulate angiogenic behaviors of HUVEC mediated by IL-8.

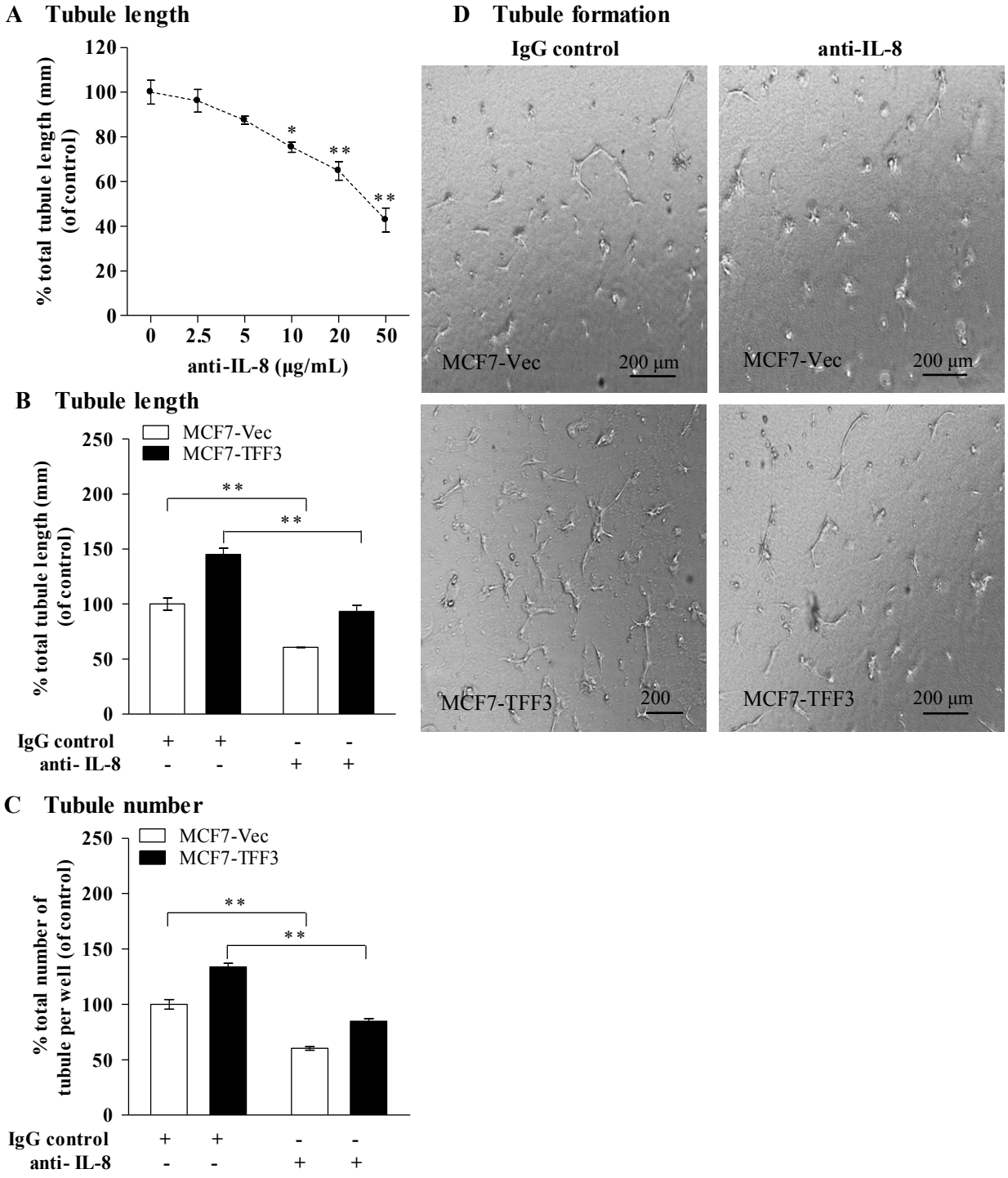

A Tubule length
% total tubule length (mm) (of control)
anti-IL-8 (μg/mL)
B Tubule length
MCF7-Vec
MCF7-TFF3
% total tubule length (mm) (of control)
IgG control
anti- IL-8
C Tubule number
% total number of tubule per well (of control)
D Tubule formation
IgG control
anti-IL-8
MCF7-Vec
MCF7-TFF3
200 μm

Figure 42: Blocking IL-8 in mammary carcinoma cells by anti-IL-8 monoclonal antibody inhibited the ability of TFF3 on stimulation of HUVEC tubule formation *in vitro*. A, HUVEC tubule formation *in vitro* in the Matrigel after 12 hours co-culture with MCF7-Vec treated with different concentration of anti-IL-8 monoclonal antibody (2.5, 5.0, 10.0, 20.0, 50 μg/mL) or IgG control in serum-free conditions. IgG was used as control. Total tubule length was assessed after 12 hours incubation. B and C, HUVEC tubule formation *in vitro* on Matrigel after 12 hours co-culture with control vector cells or MCF-7 cells with forced expression of TFF3 treated with IgG control and 50 μg/mL of anti-IL-8 monoclonal antibody in serum-free conditions. Total tubule length (B) and tubule number (C) was assessed using ImageJ analysis software. D, representative light microscopy images of HUVEC tubule formation *in vitro* in the Matrigel after 12 hours co-culture with MCF7-Vec or MCF7-TFF3 treated with IgG control rand 50 μg/mL anti IL-8 monoclonal antibody. IgG control was used as control. MCF7-Vec treated with IgG control was used as baseline. The light microscopy images were taken at x40 magnification. *, $P < 0.05$; **, $P < 0.01$ as compared to MCF7-Vec and MCF7-TFF3 treated with IgG control; scale bar, 200 μm.

3.2.14 Blocking CXCR2 in HUVEC inhibited IL-8 mediated stimulatory effect of TFF3 on HUVEC tubule formation *in vitro*

IL-8 receptors CXCR1 and CXCR2 are highly expressed in cancer and endothelial cells to mediate the angiogenic function of IL-8 in tumor angiogenesis (Holmes *et al.*, 1991, Cerretti *et al.*, 1993). Neutralizing antibody to IL-8, CXCR1 or CXCR2 has been reported to inhibit endothelial cell proliferation and capillary tube formation (Li *et al.*, 2005). To determine whether blocking CXCR2 in HUVEC inhibited tubule formation *in vitro*, parental HUVEC were treated with different concentration of anti-CXCR2 monoclonal antibody. HUVEC tubule formation *in vitro* was inhibited by anti-CXCR2 monoclonal antibody in a concentration-dependent manner, with 44% reduction of tubule length at 20 μg/mL anti-CXCR2 monoclonal antibody (Figure 43A). These data indicated that anti-CXCR2 monoclonal antibody inhibited HUVEC tubule formation *in vitro* in a concentration-dependent manner.

To determine whether the effect of TFF3 on stimulation of HUVEC tubule formation *in vitro* was mediated by IL-8 through CXCR1 or CXCR2 receptor, MCF7-Vec and MCF7-TFF3 co-cultured with HUVEC were treated with 20 μg/mL anti-CXCR2 or anti-CXCR1 monoclonal antibody respectively. Co-culturing MCF7-Vec and MCF7-TFF3 with HUVEC

treated with anti-CXCR1 monoclonal antibody exhibited similar or slight changes in tubule length and tubule number when compared with the respective cells co-cultured with HUVEC treated with IgG control (Figure 44A-C). Anti-CXCR1 monoclonal antibody presumably was not able to affect the ability of TFF3 on stimulation of HUVEC tubule formation *in vitro*. However, co-culturing MCF7-Vec with HUVEC treated with anti-CXCR2 monoclonal antibody decreased tubule length by 41% (Figure 43B) and tubule number by 47% (Figure 43C) when compared with HUVEC treated with IgG control. Co-culturing MCF7-TFF3 with HUVEC treated with anti-CXCR2 monoclonal antibody significantly decreased tubule length by 66% (Figure 43B) and tubule number by 58% (Figure 43C) when compared with HUVEC treated with IgG control. Co-culturing MCF7-Vec and MCF7-TFF3 with HUVEC treated with anti-CXCR2 monoclonal antibody decreased tubules in the Matrigel when compared with MCF7-Vec and MCF7-TFF3 co-cultured with HUVEC treated with IgG control (Figure 43D). These observations indicated that blocking of CXCR2 in HUVEC by anti-CXCR2 monoclonal antibody inhibited the effect of TFF3 on the stimulation of angiogenic behaviors of HUVEC mediated by IL-8 through CXCR2 receptor. It is also possible that the anti-CXCR2 monoclonal antibody is disrupting IL-8-independent events because CXCR2 binds to IL-8 and other chemokines.

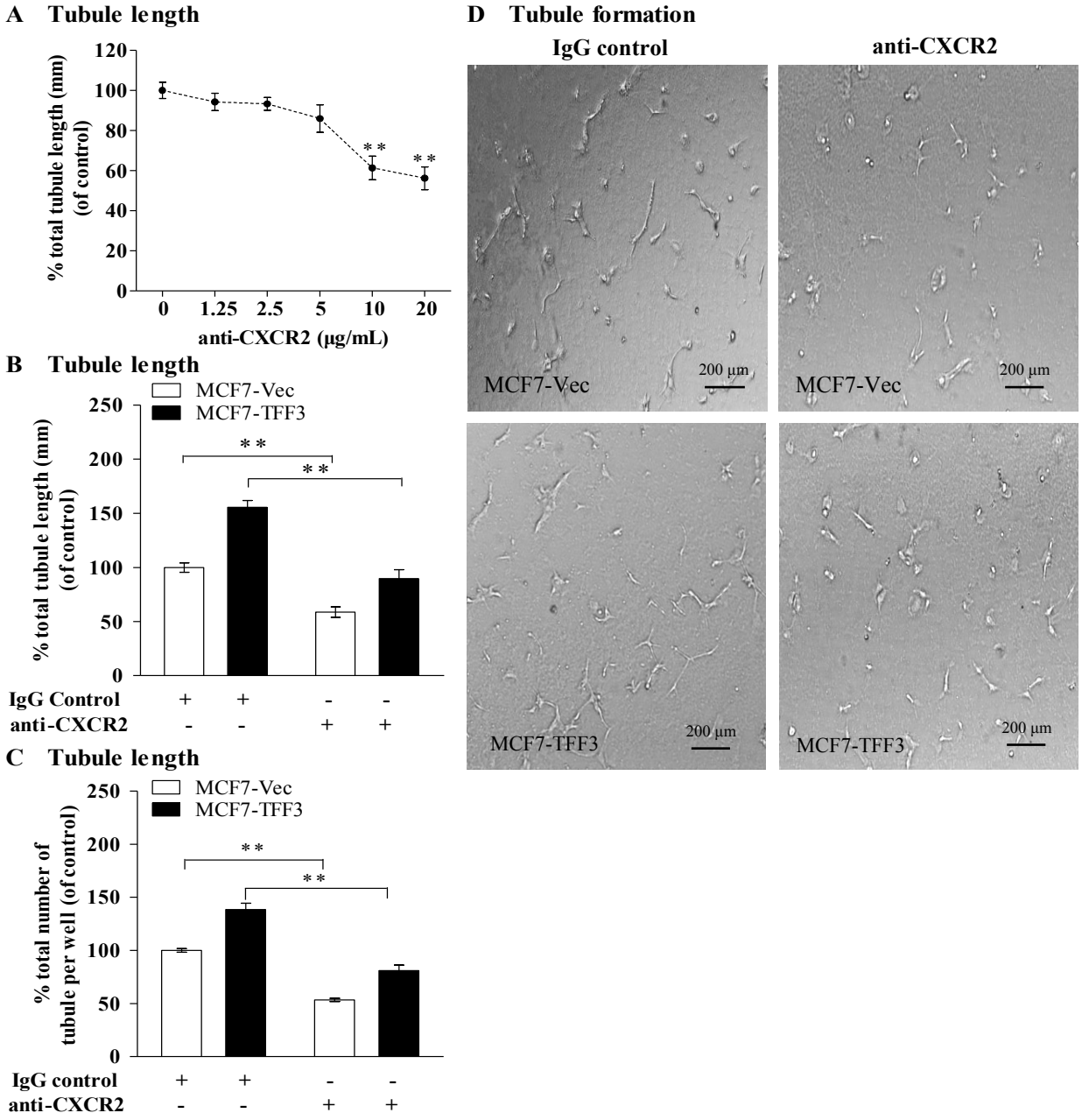


Figure 43: Blocking of CXCR2 in HUVEC by anti-CXCR2 monoclonal antibody inhibited IL-8 mediated stimulatory effect of TFF3 on HUVEC tubule formation *in vitro*. A, HUVEC tubule formation *in vitro*, in which HUVEC treated with different concentration of anti-CXCR2 monoclonal antibody (1.25, 2.5, 5.0, 10.0, 20 μg/mL) or IgG control. IgG was used as control. MCF7-Vec co-cultured with HUVEC treated with IgG control was used as baseline. Total tubule length was assessed after 12 hours incubation. B and C, HUVEC tubule formation *in vitro,* in which control vector cells or MCF-7 cells with forced expression of TFF3 co-cultured with HUVEC treated with IgG control and 20 μg/mL of anti-CXCR2 monoclonal antibody. Total tubule length (B) and tubule number (C) was assessed by ImageJ analysis software. D, representative light microscopy images of HUVEC tubule formation *in vitro*, in which MCF7-Vec and MCF7-TFF3 co-cultured with HUVEC treated with IgG control and 20 μg/mL of anti-CXCR2 monoclonal antibody. IgG was used as control. MCF7-Vec co-cultured with HUVEC treated with IgG control was used as baseline. The light microscopy images were taken at ×40 magnification. **, $P < 0.01$ as compared to HUVEC treated with IgG control; scale bar, 200 μm.

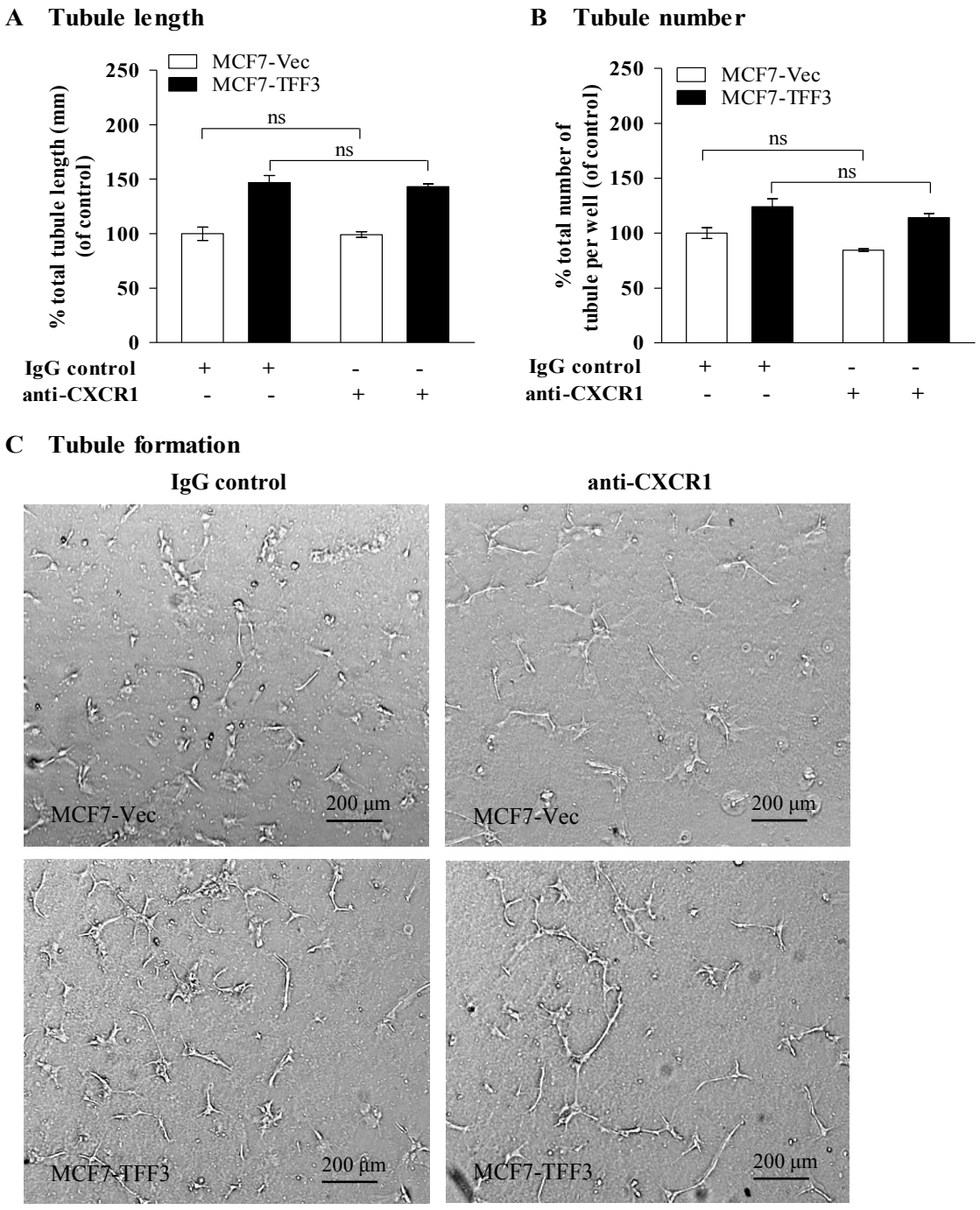


Figure 44: Blocking of CXCR1 in HUVEC by anti-CXCR1 monoclonal antibody did not affect IL-8 mediated stimulatory effect of TFF3 on HUVEC tubule formation *in vitro*. A and B, HUVEC tubule formation *in vitro*, in which control vector cells or MCF-7 cell with forced expression of TFF3 co-cultured with HUVEC treated with IgG control and 20 μg/mL anti-CXCR1 monoclonal antibody. Total tubule length (A) and tubule number (B) was assessed by ImageJ analysis software. C, representative light microscopy images of HUVEC tubule formation *in vitro*, in which MCF7-Vec and MCF7-TFF3 co-cultured with HUVEC treated with IgG control or 20 μg/mL of anti-CXCR1 monoclonal antibody. IgG was used as control. MCF7-Vec co-cultured with HUVEC treated with IgG control was used as baseline. The light microscopy images were taken at ×40 magnification. n.s indicated not significant, as compared to HUVEC treated with IgG control; scale bar, 200 μm.

3.2.15 Forced expression of TFF3 in mammary carcinoma cells promoted activation of STAT3 and subsequently partially increased IL-8 expression

In addition to the three main transcription factors NF-κB, NF-IL-6, and AP-1, TFF3 may be stimulated other transcription factors for regulation of IL-8 expression. STAT3 regulated IL-8 expression presumably through direct binding to its consensus binding site at the IL-8 promoter region (Oka *et al.*, 2010). To determine if TFF3 stimulated activation of STAT3 (tyrosine 705 phosphorylation) in MCF-7 cells, the expression of total STAT3 and Y705 phosphorylated STAT3 protein (STAT3α and STAT3β isoforms) in MCF-7 cells with forced expression of TFF3 were analyzed by Western blot analysis. When compared with control MCF7-Vec, forced expression of TFF3 in MCF-7 cells increased Y705 phosphorylated STAT3 proteins (STAT3α and STAT3β isoforms) and exhibited relatively similar level of total STAT3 protein (Figure 45A), indicating that TFF3 increased Y705 phosphorylated STAT3 protein and presumably not affected the total STAT3 protein expression.

To determine if TFF3 stimulated activation of STAT3 in MCF-7 cells and involved in the regulate IL-8 expression, STAT3 siRNA was employed to deplete STAT3 and therefore decreased STAT3 activity in mammary carcinoma cells. MCF-7 cells with forced expression of TFF3 were transiently transfected with a pcDNA containing STAT3 siRNA (siSTAT3) or pcDNA vector containing control siRNA (siControl). The expressions of total STAT3 and Y705 phosphorylated STAT3 in MCF7-Vec and MCF7-TFF3 with depletion of STAT3 were analyzed by Western blot analysis. Depletion of STAT3 in MCF-7 cells with forced expression of TFF3 decreased total STAT3 protein and Y705 phosphorylated STAT3 protein (Figure 45B) when compared with the respective cells transiently transfected with control siRNA. Subsequently, MCF7-Vec and MCF7-TFF3 with depletion of STAT3 were transiently transfected with a full length of IL-8 promoter reporter vector (-1480 to + 104 bp) and a pRL-CMV control reporter vector. Depletion of STAT3 by siRNA in MCF7-Vec and MCF7-TFF3 resulted in a small but consistently decreased of IL-8 promoter activity when

compared with the respective siControl cells (Figure 45C), indicating that STAT3 siRNA partially inhibited the effect of TFF3 on stimulation of IL-8 promoter activity. Additionally, depletion of STAT3 by siRNA partially abrogated the ability of TFF3 to regulate the expression of IL-8 and resulted in a small but consistently decreased expression of IL-8 mRNA (Figure 45D) and IL-8 protein secreted to the medium (Figure 45E) when compared with the respective siControl cells. These observations indicated that depletion of STAT3 by siRNA in MCF-7 cells partially reduced IL-8 promoter activity and subsequently led to a small decreased of IL-8 expression.

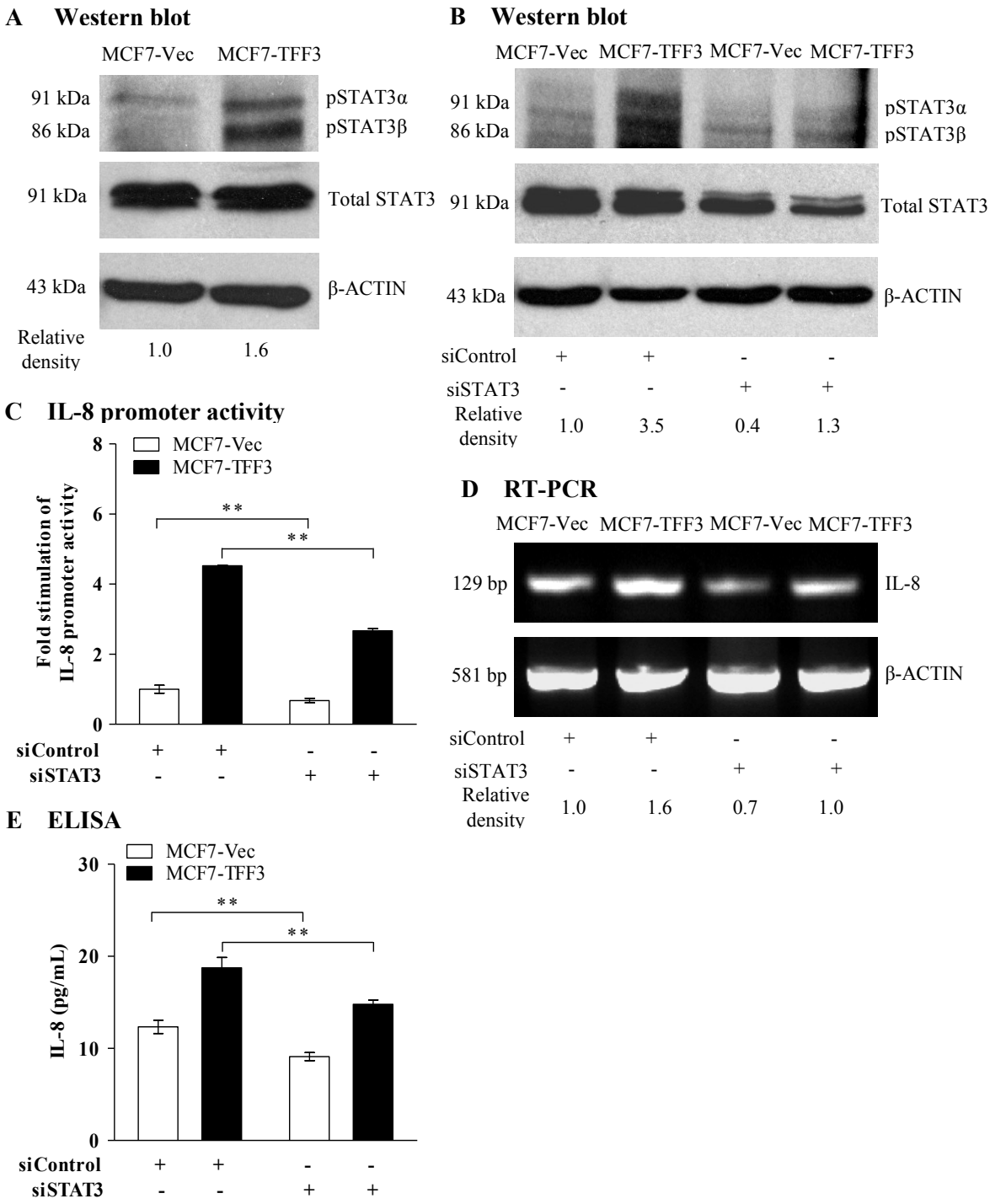


Figure 45: Forced expression of TFF3 in mammary carcinoma cells promoted the activation of STAT3 and subsequently partially increased IL-8 expression. A, Western blot analysis of total STAT3 protein and Y705 phosphorylated STAT3 (pSTAT3) in MCF-7 cells with forced expression of TFF3. B, Western blot analysis of total STAT3 and Y705 phosphorylated STAT3 protein in MCF7-Vec and MCF7-TFF3 transiently transfected with pcDNA vector containing STAT3 siRNA or control siRNA. C, IL-8 promoter activity in MCF7-Vec and MCF7-TFF3 with depletion of STAT3 transiently transfected with an IL-8 promoter reporter vector and a pRL-CMV control reporter vector. Scrambled control siRNA was used as control. MCF7-Vec transiently transfected with scrambled control siRNA was used as baseline. D, semi-quantitative analysis of IL-8 mRNA expression in MCF-7 cells with

forced expression of TFF3 transiently transfected with pcDNA vector containing STAT3 siRNA or control siRNA. E, ELISA analysis of IL-8 protein secreted to the medium by MCF7-Vec and MCF7-TFF3 transiently transfected with pcDNA vector containing STAT3 siRNA or control siRNA. β-ACTIN was used as a loading control in semi-quantitative RT-PCR and Western blot analysis. **, $P < 0.01$ as compared MCF7-Vec or MCF7-TFF3 transiently transfected with control siRNA.

3.2.16 Depletion of STAT3 by siRNA partially abrogated stimulatory effect of TFF3 on HUVEC migration, invasion, and tubule formation *in vitro*

To determine if STAT3 mediated TFF3 enhancement of angiogenic behaviors of endothelial cells, HUVEC were co-culture with MCF7-Vec and MCF7-TFF3 with depletion of STAT3. Depletion of STAT3 by siRNA in MCF7-Vec partially decreased HUVEC migration by 36% (Figure 46A) and invasion by 31% (Figure 46B) when compared with MCF7-Vec transiently transfected with control siRNA. Depleted expression of STAT3 in MCF7-TFF3 partially decreased HUVEC migration by 47% (Figure 46A) and invasion by 58% (Figure 46B) when compared with MCF7-TFF3 transiently transfected with control siRNA. These observations indicated that depletion of STAT3 by siRNA partially abrogated the ability of TFF3 to stimulate HUVEC migration and invasion.

Furthermore, depletion of STAT3 in MCF7-Vec significantly decreased tubule length by 32% (Figure 46C) and tubule number by 31% (Figure 46D) when compared with MCF7-Vec transiently transfected with control siRNA. Depletion of STAT3 in MCF7-TFF3 significantly decreased tubule length by 46% (Figure 46C) and tubule number by 58% (Figure 46D) when compared with MCF7-TFF3 transiently transfected with control siRNA. Depleted expression of STAT3 by siRNA in MCF7-Vec and MCF-TFF3 significantly decreased tubules formed by the HUVEC when compared with the respective siControl cells (Figure 46E). These observations indicated that depletion of STAT3 by siRNA partially abrogated the ability of TFF3 on stimulation of HUVEC tubule formation *in vitro*.

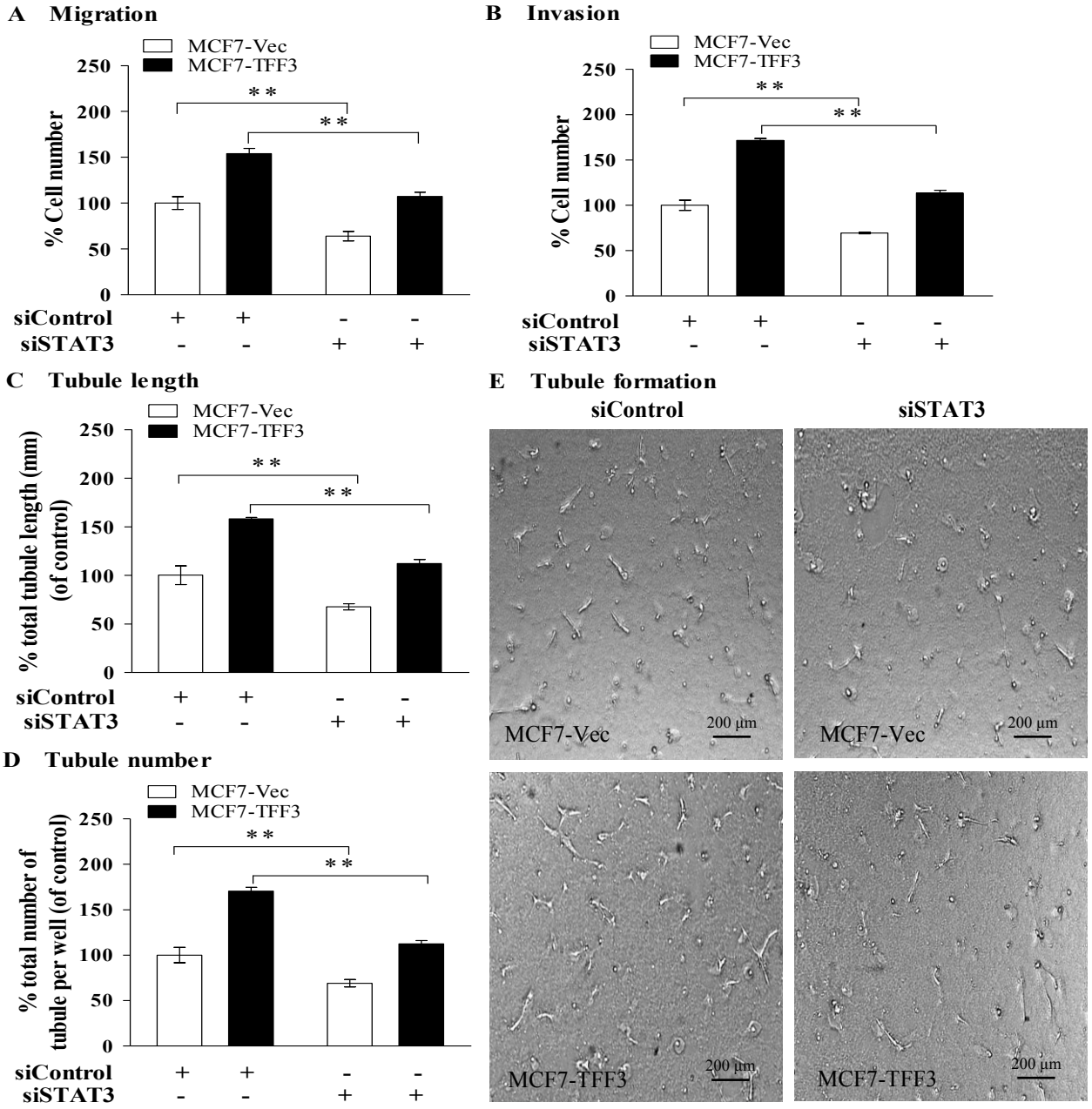


Figure 46: Depletion of STAT3 in mammary carcinoma cells by siRNA partially abrogated stimulatory effect of TFF3 on HUVEC migration, invasion, and tubule formation *in vitro*. A, HUVEC migration after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 transiently transfected with STAT3 siRNA. Scrambled control siRNA was used as control. MCF7-Vec transiently transfected with scrambled control siRNA was used as baseline. B, HUVEC invasion after 24 hours co-culture with MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or STAT3 siRNA. C and D, HUVEC tubule formation *in vitro* in the Matrigel after 12 hours co-culture with MCF-7 cells with forced expression of TFF3 transiently transfected with control siRNA or STAT3 siRNA in serum-free conditions. Total tubule length (C) and tubule number (D) was assessed. E, representative light microscopy images of HUVEC tubule formation *in vitro* in the Matrigel after 12 hours co-culture of with MCF7-Vec and MCF7-TFF3 transiently transfected with control siRNA or STAT3 siRNA. The light microscopy images were taken at ×40 magnification. **, $P < 0.01$ as compared to MCF7-Vec or MCF7-TFF3 transiently transfected with control siRNA; scale bar, 200 µm.

3.3 Discussion

3.3.1 Forced expression of TFF3 in mammary carcinoma cells promoted de novo angiogenesis

TFF3 has been reported increased its expression in mammary carcinoma and the expression of TFF3 is positively associated with metastasis and poor survival outcome of patients with ER+ mammary carcinoma (Smid *et al.*, 2006, Kannan *et al.*, 2010, Pandey *et al.*, 2014). Increased TFF3 expression in mammary carcinoma cells promotes tumor growth and metastatic progression by promoting cell survival and invasion (Kannan *et al.*, 2010, Ahmed *et al.*, 2012, Pandey *et al.*, 2014). It is known that TFF3 is an estrogen-responsive gene and its expression is associated with the ER status in human mammary carcinoma (May and Westley, 1997a, Gruvberger *et al.*, 2001). However, there is a subset of ER- mammary carcinoma cells that express high levels of TFF3 (Doane *et al.*, 2006). Herein, I have demonstrated that ER+ mammary carcinoma cells endogenously expressed TFF3 protein. Both MCF-7 and T47D cells expressed moderate levels of TFF3 and were utilized as mammary adenocarcinoma cell models. HUVEC was used as an *in vitro* model to investigate the stimulatory effect of TFF3 secreted from mammary carcinoma cells on the angiogenic behaviors of endothelial cells. TFF3 secreted from mammary carcinoma cells promoted HUVEC monolayer proliferation, cell cycle progression, survival, migration, invasion and tubule formation *in vitro*. Depletion of TFF3 in mammary carcinoma cells by siRNA diminished angiogenic behaviors of HUVEC.

During neovascularization, endothelial cells invade to avascular environments to generate vasculature in the tumor (Hanahan and Folkman, 1996). The survival of endothelial cells is one of the major factors for maintaining the stability of vascularity in the growing tumor (Nor *et al.*, 1999). It has been reported that TFF3 decreased apoptotic cell death of MCF-7 cells by approximately 10% in serum free conditions (Kannan *et al.*, 2010). I have demonstrated that TFF3 secreted from MCF7 cells decreased HUVEC apoptotic cell death by 61% in 10%FBS condition and 52% in serum free conditions. Similarly, Artemin secreted from mammary carcinoma cells decreased apoptotic cell death of endothelial cells in serum

deprived condition by 20% (Banerjee *et al.*, 2012). It is possible that the survival of endothelial cells was not affected by the supplemental function of serum but is dependent on the pro-survival effect of TFF3. I have demonstrated that TFF3 secreted by mammary carcinoma cells with forced expression of TFF3 promoted HUVEC proliferation and survival. TFF3 may also a pro-survival factor for endothelial cells. It has previously been reported that TFF3 is a pro-survival factor for cancer cells (Chen *et al.*, 2000, Taupin *et al.*, 2000b, Regalo *et al.*, 2005, Kannan *et al.*, 2010) and induced resistance to serum deprivation and drug-induced apoptosis in colon carcinoma cells (Emami *et al.*, 2004). Forced expression of TFF3 increased Bcl-2 (Kannan *et al.*, 2010), which is an anti-apoptotic protein (Yip and Reed, 2008) and decreased Bax, which is a pro-apoptotic Bcl-2-family proteins (Yip and Reed, 2008, Czabotar *et al.*, 2009). Increased the expression of Bcl-2 mediated TFF3-stimulated resistance to serum deprivation and doxorubicin-induced apoptosis in mammary carcinoma cells (Kannan *et al.*, 2010). TFF3 appears to be linked with multiple survival pathways including mitogen-activated protein kinase (MAPK) (Taupin *et al.*, 1999), phosphatidylinositol-3-kinase-Akt (PI3K-Akt) (Kinoshita *et al.*, 2000b, Taupin *et al.*, 2000b), STAT3 (Rivat *et al.*, 2005) and nuclear factor kappa B (NF-κB) (Chen *et al.*, 2000). Enhanced Bcl2 expression is probably a consequence of activation of survival pathways, resulting from increased TFF3 expression (Kannan *et al.*, 2010). Additionally, I have demonstrated that forced expression of TFF3 in mammary carcinoma cells promoted HUVEC migration and invasion. TFF peptides induced cellular invasion of kidney and colonic carcinoma cells through autocrine loops (Emami *et al.*, 2001). TFF3 also promoted colon carcinoma cell migration through repression of E-cadherin-catenin complex formation and loss of cell adhesion (Emami *et al.*, 2004).

De novo angiogenesis is a fundamental requirement for tumor growth and metastasis of mammary carcinoma. High microvessel density has been associated with metastasis and poor survival in node-negative mammary carcinoma (Schneider and Miller, 2005, Fox *et al.*, 2007, Ran *et al.*, 2010). TFF3 has been implicated in tumor progression by promoting cell scattering and cellular invasion (Rodrigues *et al.*, 2003b). Increased the expression of TFF3

was positively correlated with microvessel density as evaluated by CD31 and CD34 of endothelial markers in gastric carcinoma (Dhar *et al.*, 2005) and mammary carcinoma (Ahmed *et al.*, 2012). Concordantly, I have demonstrated that TFF3 secreted from mammary carcinoma cells promoted HUVEC tubule formation *in vitro*. Hence, TFF3 secreted from mammary carcinoma cells stimulated angiogenic behaviors of HUVEC to promote *in vitro* angiogenesis. In xenograft models, TFF3 enhanced microvessel density (increased areas of CD31 and CD34 labeled cells) in the tumors produced by MCF-7 cells and suggesting that TFF3 promoted *in vivo* angiogenesis. These observations further supported that TFF3 promotes *de novo* angiogenesis in mammary carcinoma and therefore TFF3 is as a promoter of angiogenesis.

3.3.2 The mechanism by which TFF3 promoted *de novo* angiogenesis in mammary carcinoma cells

(i) IL-8 mediated angiogenic behaviors of endothelial cells stimulated by TFF3

Herein, I have demonstrated that TFF3 may not enhance VEGF-A expression in mammary carcinoma cells, suggesting that VEGF-A probably may not involve in modulation of TFF3 to stimulate angiogenic behaviors of HUVEC. TFF3 promoted *de novo* angiogenesis in mammary carcinoma is probably independent of VEGF-A signaling. These observations were unexpected because several reports have suggested that the angiogenic responses of VEGF-A are mediated by VEGFR2 receptor and it also stimulated activation of COX-2 or EGF-R tyrosine kinase to exert its angiogenic functions controlled by TFF peptides (Rodrigues *et al*., 2003). It is well-known that VEGF-A plays a dominant role in tumor angiogenesis and is a potent angiogenic cytokine. VEGF-A promotes proliferation, migration, and survival of endothelial cells (Ferrara *et al.*, 2003, Ferrara, 2005). Increased VEGF-A expression is associated with tumor angiogenesis and poor prognosis of patient with breast cancer (Schneider and Miller, 2005). TFF peptides are not acting through VEGF-A/VEGFR signaling pathway because VEGFR2 tyrosine kinase inhibitor was not effectively attenuated

invasion of colon cancer cells stimulated by TFF3 (Rivat *et al.*, 2005). Conversely, depletion of STAT3 by siRNA abrogated cellular invasion promoted by TFF3 and VEGF-A, suggesting that the autocrine self-activation of TFF3 and VEGF-A in colon cancer cells are dependent on STAT3 signal transduction pathway (Rivat *et al.*, 2005). Concordantly, TFF3 stimulated phosphorylation of c-Src and subsequently increased STAT3 activity which led to down-regulation of E-cadherin for promoting invasion and metastasis of mammary carcinoma (Pandey *et al.*, 2014).

Herein, I have demonstrated that forced expression of TFF3 in mammary carcinoma cells increased IL-8 promoter activity and subsequently enhanced IL-8 expression. In xenograft model, mammary carcinoma cells with forced expression of TFF3 produced tumor with increased IL-8 protein expression. Forced expression of TFF3 in mammary carcinoma cells increased IL-8 expression, which in turn mediated the stimulatory effect of TFF3 in promoting *de novo* angiogenesis of mammary carcinoma. It has been reported that chemokine and its cognate receptors play an important role in facilitating invasion, angiogenesis, and metastasis of cancer cells (Richmond *et al.*, 2009, Singh *et al.*, 2010). IL-8 directly promoted angiogenesis via stimulation of endothelial cell proliferation, migration, survival and tubule formation (Koch *et al.*, 1992, Hu *et al.*, 1993, Heidemann *et al.*, 2003, Snyder *et al.*, 2004, Ning *et al.*, 2010). Increased IL-8 expression is associated with enhancing invasion and angiogenesis in mammary carcinoma (Lin *et al.*, 2004). Furthermore, IL-8 has been suggested as a metastatic factor in mammary carcinoma to regulate angiogenesis, tumor growth, and dissemination of cancer cells (Murdoch *et al.*, 1999, Salcedo *et al.*, 2000b). An *in vivo* study showed that the basal level of IL-8 expression is substantially higher in the estrogen-independent mammary carcinoma cell lines (e.g. MDA-MB-231) when compared with the estrogen-dependent mammary carcinoma cell lines (e.g. MCF-7), demonstrating a positive correlation between IL-8 expression and the metastatic capacity of mammary carcinoma cell lines (De Larco *et al.*, 2001, Lin *et al.*, 2004). In a tumor xenograft model, IL-8 expressing cells formed larger tumors than the control cells with enhanced microvessel density and

increased IL-8 expression was associated with the tumor growth and angiogenesis of breast cancer (Lin *et al.*, 2004, Zhou *et al.*, 2006).

In addition to metastatic potential of IL-8, the expression of TFF3 in mammary carcinoma is an predictive biomarker for metastasis (Ahmed *et al.*, 2012). TFF3 has also been identified as a promoter of metastatic dissemination of mammary carcinoma cells (Pandey *et al.*, 2014). The expression of TFF3 is positively associated with the metastatic phenotype in mammary carcinoma cells. Increased expression of TFF3 is associated with invasion of muscle, the nipple, neural sheath, and the lymphovasculature (Ahmed *et al.*, 2012). Increased TFF3 expression stimulated mammary carcinoma cells to metastasize away from the primary tumor and penetrated into the lymphovasculature followed by forming of micrometastatic breast cancer (Ahmed *et al.*, 2012).

I have observed that mammary carcinoma cells with forced expression of TFF3 expressed CXCR1 and CXCR2 mRNAs. Numerous of studies have demonstrated that the differential expression of IL-8 receptors between benign and malignant breast cancer tissues. A clinical study by Miller *et al.* (1998) reported that all breast cancer cells expressed IL-8 receptors, CXCR1 and CXCR2. In contrast, Freund *et al.* (2003) observed that CXCR1 expression is extremely low in mammary carcinoma cell lines, but most of the cells expressed CXCR2, without any correlation with ER status (Freund *et al.*, 2003). Notably, IL-8 receptors expressed in many human cancer cells with no apparent correlation with the grade of the tumor (Miller *et al.*, 1998, Muller *et al.*, 2001).

The tumor promoting effects of IL-8 in modulating the survival, angiogenesis, and metastasis of cancer cells were attenuated by humanized anti-IL-8 monoclonal antibodies (Fujisawa *et al.*, 2000, Li *et al.*, 2003a). Depletion of IL-8 in human melanoma cell lines by anti-sense RNA has revealed that IL-8 may function as a growth modulator (Schadendorf *et al.*, 1993). Administration of humanized monoclonal antibodies against IL-8 (e.g., ABX-IL-8) attenuated the growth of bladder cancer (Mian *et al.*, 2003) and decreased tumorigenic and metastatic potential of melanoma in xenograft models (Huang *et al.*, 2002). IL-8 gene

silencing by siRNA has been exploited to deplete IL-8 expression in ovarian tumor xenograft models, resulting in growth retardation and reduced microvessel density (Merritt *et al.*, 2008). Concordantly, I have demonstrated that depletion of IL-8 in mammary carcinoma cells by siRNA or inhibition of IL-8 with anti-IL-8 monoclonal antibody abrogated the ability of TFF3 to stimulate angiogenic behaviors of HUVEC. Additionally, inhibition of IL-8 in mammary carcinoma cells by anti-IL-8 monoclonal antibody attenuated the effect of TFF3 on the stimulation of HUVEC tubule formation *in vitro* mediated by IL-8. Both IL-8 siRNA and anti-IL-8 monoclonal antibody were able to inhibit the ability of TFF3 to stimulate angiogenic behaviors of HUVEC for promoting *de novo* angiogenesis in mammary carcinoma.

IL-8 receptors in the endothelial cells have been shown to be involved in tumor angiogenesis (Singh *et al.*, 2010). IL-8 binds to its cognate receptors either CXCR1 or CXCR2 and subsequently promotes endothelial cell proliferation and tumor angiogenesis. Administration of neutralizing antibodies against to CXCR1 and CXCR2 in endothelial cells abrogated tubule formation stimulated by IL-8, indicating that these two receptors are critical for activation of angiogenic responses of IL-8 (Salcedo *et al.*, 2000a, Li *et al.*, 2005). Other studies have also supported the importance of CXCR1 and CXCR2 in tumor angiogenesis (Varney *et al.*, 2006, Singh *et al.*, 2009). Of these two high affinity receptors, CXCR2 receptor in the endothelial cells is more prone to implicate in tumor angiogenesis stimulated by IL-8 (Addison *et al.*, 2000, Strieter *et al.*, 2004). I have observed that blocking of CXCR1 in the HUVEC by anti-CXCR1 monoclonal antibody was probably not able to inhibit the effect of TFF3 on the stimulation of HUVEC tubule formation *in vitro*. However, blocking of CXCR2 in the HUVEC by anti-CXCR2 monoclonal antibody abrogated the ability of TFF3 to stimulate HUVEC tubule formation *in vitro* mediated by IL-8 through CXCR2. It is known that CXCR2 has other chemokine ligands such as IL-1, IL-2 and IL-6 (Addison *et al.*, 2000). It is possible that anti-CXCR2 is disrupting IL-8-independent events. These observations were concordant with Heidemann *et al.* (2003) that IL-8 promoted angiogenesis in the human intestinal microvascular endothelial cells is dependent on CXCR2, a receptor targeted by

multiple angiogenic CXC chemokines. Other studies have also reported that the migration of endothelial cells can be suppressed by neutralizing antibody to CXCR2 (Schraufstatter *et al.*, 2001, Li *et al.*, 2005) and delayed vascularization in CXCR2 knockout mice (Devalaraja *et al.*, 2000). Therefore, I suggested that TFF3 stimulates angiogenic behaviors of HUVEC for promoting *de novo* angiogenesis in mammary carcinoma mediated by IL-8 through CXCR2 axis.

(ii) STAT3 partially mediated angiogenic behaviors of endothelial cells stimulated by TFF3

IL-8 promoter activity is regulated by the differential binding of the transcription factors namely NF-IL-6, NF-κB, and AP-1 (Mukaida *et al.*, 1990, Roebuck, 1999) and several other nuclear factors such as hepatocyte nuclear factor-1 and glucocorticoid-responsive element (Mukaida *et al.*, 1989). The regulatory elements in the proximal region of IL-8 gene are important for activation of IL-8 promoter activity (Kashima *et al.*, 2009). The 5'-flanking region of the IL-8 contains DNA response elements for transcription factors NF-IL-6 (-94 to -81 bp), AP-1 (-126 to 120 bp) and NF-κB (-80 to -70 bp) (Mukaida *et al.*, 1994). These three transcription factors binding sites are the major elements involved in the regulation of the IL-8 and their physical proximity may facilitate cooperative activation of these elements (Roebuck, 1999). Concordantly, I have demonstrated that TFF3 stimulated IL-8 promoter activity and subsequently increased IL-8 expression. Additionally, the transcription factors namely NF-IL-6, AP-1 and NF-κB, present at the proximal region of IL-8 promoter, were involved in enhancing TFF3-stimulated IL-8 promoter activity. Apparently, multiple transcription factors are involved in the regulation of IL-8 stimulated by TFF3.

The production of IL-8 is modulated by activation of IL-8 promoter via cooperation of multiple transcription factors (Le *et al.*, 2000). It has been reported that increased IL-8 expression was predominantly stimulated by NF-κB (Karashima *et al.*, 2003). Constitutive IL-8 expression required both NF-κB and STAT3, over-expression of activated STAT3 resulted in a drastically increase of IL-8 protein expression (Scholz *et al.*, 2003). The

Src/STAT3 pathways may promote IL-8 production independent of NF-κB (Trevino *et al.*, 2006). Gharavi *et al.* (2007) suggested that either STAT3 alone is adequate or a different co-activator such as NF-κB is required to cooperate with STAT3 for stimulation of IL-8 expression in cancer cells (Gharavi *et al.*, 2007). STAT3 is an important regulatory element involved in the regulation of IL-8 and STAT3 presumably direct interaction with its consensus binding site at the IL-8 promoter region (Oka *et al.*, 2010). Concordantly, Seidel *et al*. (1995) has reported that a putative STAT3 binding site was predicted in the 5'-flanking region of IL-8 gene (Seidel *et al.*, 1995).

Herein, I have demonstrated that forced expression of TFF3 in mammary carcinoma cells increased tyrosine phosphorylation of STAT3 protein (Y705 pSTAT3). This observation was consistent with a recent study shown that TFF3 promoted STAT3 activity in mammary carcinoma cells through activation of c-Scr protein (Pandey *et al.*, 2014). Depletion of STAT3 by siRNA partially abrogated the ability of TFF3 to stimulate IL-8 promoter activity and resulted in a small decreased of IL-8 expression. Additionally, depletion of STAT3 in mammary carcinoma cells by siRNA partially abrogated the ability of TFF3 to stimulate angiogenic behaviors of HUVEC mediated by STAT3. Furthermore, TFF3 was shown to promote a mesenchymal and invasive (EMT) phenotype to enhance the metastatic capability of mammary carcinoma cells through c-Src/STAT3 signaling pathway by activation of EMT markers (Pandey *et al.*, 2014). Thereby, forced expression of TFF3 down-regulated the expression of E-cadherin and concomitantly increased expression of vimentin to promote metastatic dissemination of mammary carcinoma cells (Pandey *et al.*, 2014).

A number of reports have shown that STAT3 plays a pivotal role in tumor angiogenesis by enhancing VEGF protein expression (Niu *et al.*, 2002, Osugi *et al.*, 2002, Schaefer *et al.*, 2002). STAT3 activity is regulated by post-translational modifications involving phosphorylation of specific conserved tyrosine residues (e.g., tyrosine 705) and active nuclear translocation followed by DNA binding and transcriptional activation of the target genes (Jove, 2000). The constitutively active STAT3 directly acts on VEGF-A gene to

stimulate VEGF-A promoter activity and subsequently up-regulates VEGF-A expression to stimulate neovascularization in the tumors (Niu *et al.*, 2002). Similarly, STAT3 may be able to directly act on the IL-8 promoter to regulate IL-8 expression. Several studies have suggested that IL-8 promoter may be containing a putative consensus binding site for STAT3, which is involved in the regulation of IL-8 (Seidel *et al.*, 1995, Oka *et al.*, 2010). Presumably, TFF3 secreted from mammary carcinoma cells stimulated tyrosine phosphorylation of STAT3, the activated STAT3 dimerizes, translocates into the nucleus, and directly binds to the IL-8 promoter for regulation of IL-8 gene transcription (Bromberg *et al.*, 1999, Garcia *et al.*, 2001, Niu *et al.*, 2002, Rivat *et al.*, 2005). Mechanistically, I suggested that forced expression of TFF3 in mammary carcinoma cells elevated IL-8 expression and increased the phosphorylated STAT3 activity. Subsequently, TFF3 stimulated angiogenic behaviors of endothelial cells for promoting *de novo* angiogenesis in mammary carcinoma through IL-8/CXCR2 axis. Hence, TFF3 is a promoter of angiogenesis, which may also co-coordinate with the growth promoting and metastatic actions of TFF3 in mammary carcinoma to enhance tumor progression.

CHAPTER 4

Exogenous Human Recombinant TFF3 and TFF3 Secreted from HUVEC Promoted Angiogenesis

4.1 Introduction

Angiogenesis is a complex and sequential process in which the existing blood vessel generates new vasculature. Following stimulation of endothelial cells by angiogenic factors, the endothelium is destabilized, producing a decrease in endothelial cell adhesion and a concomitant increase in vascular permeability (Petreaca *et al.*, 2007). The basal lamina of blood vessel is then degraded by matrix metalloproteinases to allow the endothelial cells to proliferate and migrate into surrounding connective tissues. The endothelial cells then sprout from adjacent vessel and fuse to produce a labyrinth of new blood vessels (Petreaca *et al.*, 2007). The nascent vessel recruits periendothelial and smooth muscle-like cells to stabilize the endothelium by promotion of the depositions of basal lamina and intercellular adhesion (Daniel and Abrahamson, 2000, Conway *et al.*, 2001). Angiogenesis is critical for different physiological and pathophysiological processes, including embryonic development, wound healing and chronic inflammation, and cancer progression (Folkman, 1995).

Under normal physiological conditions, angiogenesis is a tightly regulated process in which quiescent endothelial cells are induced to divide, leading to the spread of the vascular network, which supplies oxygen and nutrients to the growing tissues. Normal angiogenesis occurs during embryogenesis where it establishes the primary vasculature and generates adequate blood vessels for development of organs (Folkman, 1995). *De novo* angiogenesis is important in normal physiological repair mechanisms such as wound healing (Klagsbrun and D'Amore, 1991). The angiogenic switch in cancer is coordinated by the combinatorial balance between pro- and anti-angiogenic factors, produced either by cancer cells or by stromal cells in the surrounding interstitium (Baeriswyl and Christofori, 2009).

It is already known that angiogenic factors derived from a tumor are implicated in *de novo* angiogenesis, but there is evidence showed that angiogenic factors and the endothelial cells are pivotal origins of signals to non-vascular tissue cells during development of organs (Crivellato, 2011). Endothelial cells produce a number of secreted angiogenic proteins that enhance migration, survival and function of neighboring cells (e.g. mature endothelial cells), thereby promoting neovascularization. Furthermore, endothelial cells are crucial participants in tumor angiogenesis (Harvey *et al.*, 2002).

The expression of the TFF family of protein has been observed in tissues containing mucus-secreting cells as exemplified by the gastrointestinal tract, and colon, suggesting that their physiological functions may be related to that of mucin (Kraljevic Pavelic *et al.*, 2011). Importantly, these peptides are involved in the protection of the gastrointestinal tract against mucosal damage and in mucosal restitution (Taupin and Podolsky, 2003). In addition, TFF peptides are expressed in other organs including lymphoid tissue (Cook *et al.*, 1999), brain (Probst *et al.*, 1996, Jagla *et al.*, 2000), liver and gall bladder (Sasaki *et al.*, 2007). Thus, the functions of TFF peptides are cell-type specific. TFF peptides are secreted from diverse organs and exert different functional effects, yet their biological significance for most part is still elusive (Kjellev, 2009). *In vitro* experimental data have shown that exogenous recombinant human TFF3 (rhTFF3) stimulated migration of intestinal, respiratory and corneal epithelial cells in wound healing assays (Chinery and Playford, 1995, Playford *et al.*, 1995, Göke *et al.*, 2001, Oertel *et al.*, 2001, Graness *et al.*, 2002). Extracellular administration of rhTFF3 provides protection against ethanol-induced gastric mucosal damage and drug-induced intestinal mucosal injury (Kindon *et al.*, 1995, Playford *et al.*, 1996, McKenzie *et al.*, 2000, Babyatsky *et al.*, 2009). Furthermore, functional studies of TFF3 on epithelial mucosal damage have shown that rhTFF3 potently stimulated the migration of intestinal epithelial cells, promoted wound healing, and altered E-cadherin expression to enhance the migration of epithelial cells (Dignass *et al.*, 1994, Playford *et al.*, 1995).

A study by Rodrigues *et al.* (2003b) demonstrated that exogenous rhTFF3 promoted capillary vessel formation in a CAM assay, suggesting that TFF3 may function as pro-angiogenic factor. Moreover, the angiogenic activity of TFF1 in HUVEC tubule formation *in vitro* was comparable to that stimulated by VEGF-A. TFF1 promoted angiogenesis *in vitro* which mediated by both cyclooxgenase-2 (COX-2) and epidermal growth factor receptor (EGF-R) (Rodrigues *et al.*, 2003b). Given that TFF3 is analogous to TFF1, TFF3 may possess similar pro-angiogenic characteristic as TFF1, suggesting that TFF3 may also be involved in angiogenesis *in vivo*, although further studies are required to determine whether TFF3 can directly act on endothelial cells to stimulate its angiogenic behaviors.

HUVEC are preferable to use as a cell-based model for study of angiogenesis *in vitro* as they retain the traits or functional characteristics of normal human endothelial cells (Ades *et al.*, 1992, Bouis *et al.*, 2001). HUVEC possesses the ability to form tubule in the basement membrane extract Matrigel resembles to the ECM, thus mimicking angiogenesis *in vitro* (Donovan *et al.*, 2001, Auerbach *et al.*, 2003, Bagley *et al.*, 2003). HUVEC tubule formation *in vitro* is a simple, feasible and reproducible assay and commonly used to determine the angiogenic potential an angiogenic factor (Okamura *et al.*, 1992, Ito *et al.*, 1995). HUVEC are grown in the Matrigel in the presence of a tested angiogenic protein and subsequently lead to either promotion or inhibition of tubule formation, indicating that the angiogenic protein can directly stimulate or inhibit angiogenic behaviors of HUVEC (Aranda and Owen, 2009). The angiogenic potential of an angiogenic protein can be quantified by measuring tubule length and/or tubule number (Jones *et al.*, 1998).

In this study, the immortalized human umbilical vein endothelial cells (HUVEC) were utilized as an *in vitro* endothelial model. HUVEC with forced expression of TFF3 were generated by transient transfection with a pIRESneo3 (Invitrogen, Carlsbad, CA) expression vector containing human TFF3 cDNA (NM_003226) (designated as HUVEC-TFF3), or the pIRESneo3 empty vector (designated as HUVEC-Vec). HUVEC with depletion of TFF3 were generated by transient transfection of HUVEC with a pSilencer 2.1-U6 hygro (Ambion,

Austin, TX) expression vector containing TFF3 siRNA (designated as HUVEC-siTFF3), or with control siRNA encoding a siRNA that has no significant sequence similarity to human gene sequences (designated as HUVEC-siVec).

I therefore postulate that exogenous rhTFF3 directly acts on HUVEC to promote angiogenic behavior of HUVEC and TFF3 secreted from HUVEC may also exert similar functional effects as exogenous rhTFF3 in promoting of angiogenesis.

4.2 Results

4.2.1 Exogenous rhTFF3 increased HUVEC monolayer proliferation

Recombinant human TFF3 (rhTFF3) was expressed from pGEX-4T1-TFF3 plasmid in *E coli* and purified using glutathione-S-transferase tag affinity chromatography under non-reducing condition as previously described (Amiry *et al.*, 2009). The specificity and purity of this protein has been determined by Western blot analysis, whereby a single band was detected as a monomer at 7 kDa or dimer at 14 kDa under reducing and non-reducing conditions, respectively (Amiry *et al.*, 2009).

Different concentrations of rhTFF3 (0.1, 1.0, 2.5, 5.0, and 10.0 ng/mL) were used to examine the effect of exogenous rhTFF3 stimulation on HUVEC monolayer proliferation. The effect of exogenous rhTFF3 on HUVEC monolayer proliferation (total cell number) after 48 hours with different concentration of rhTFF3 in 0.2% FBS (serum deprived) and 10% FBS (serum replete) conditions were determined. Exogenous rhTFF3 stimulation significantly increased HUVEC monolayer proliferation from 35% at 2.5 ng/mL rhTFF3 to 100% at 10.0 ng/mL rhTFF3 in 10% FBS conditions when compared with HUVEC treated with BSA control protein (Figure 47A). Lower concentration of exogenous rhTFF3 may not able to stimulate HUVEC monolayer proliferation in serum deprived conditions (0.2% FBS), but slightly increased HUVEC monolayer proliferation by 18% at a higher concentration of rhTFF3 (10 ng/mL) (Figure 47B), indicating that exogenous rhTFF3 promoted HUVEC monolayer proliferation in a concentration-dependent manner.

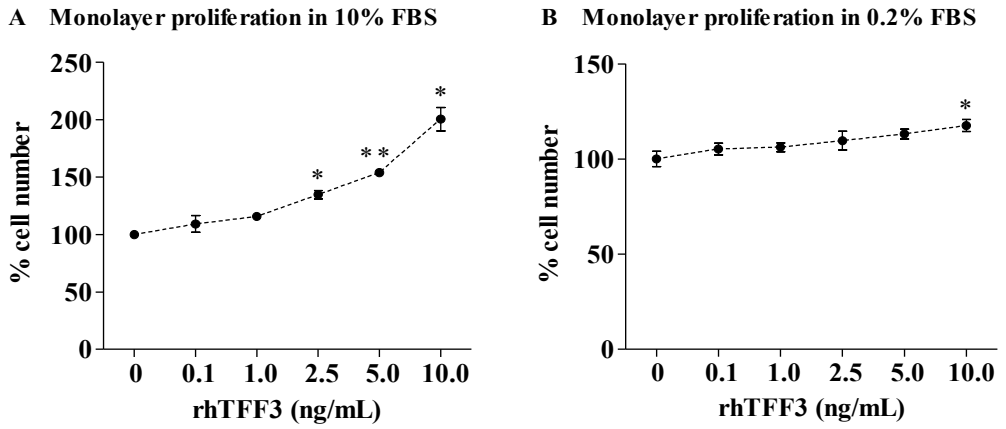


Figure 47: Exogenous rhTFF3 stimulation increased monolayer proliferation of HUVEC. A, HUVEC monolayer proliferation at different concentrations of rhTFF3 (0.1, 1.0, 2.5, 5.0, 5, 10 ng/mL of rhTFF3) in 10% FBS condition. B, HUVEC monolayer proliferation at different concentrations of rhTFF3 (0.1, 1.0, 2.5, 5.0, 5, 10 ng/mL of rhTFF3) in 0.2% FBS condition. Different concentrations of rhTFF3 in 0.2% FBS or 10% FBS EBM media was added to the HUVEC and incubated at 37^{o}C for 48 hours. BSA was used as control. HUVEC treated with 10 ng/mL BSA was used as baseline. *, $P < 0.05$; **, $P < 0.01$ as compared with BSA control.

4.2.2 Exogenous rhTFF3 stimulation increased HUVEC migration, invasion, and tubule formation *in vitro*

To determine whether the exogenous rhTFF3 stimulation acted as a chemoattractant or stimulant, a transwell migration assay was performed to examine the effect of exogenous rhTFF3 stimulation on HUVEC migration. The untreated HUVEC plated in the membrane of the transwell insert incubated with 5.0 ng/mL rhTFF3 in the bottom well of the companion plate increased HUVEC migration as compared to the HUVEC incubated with BSA control and therefore rhTFF3 acted as a chemoattractant (Figure 48A). Alternatively, HUVEC treated with 5 ng/mL rhTFF3 plated in the membrane of transwell insert and incubated with 10% FBS EBM medium in the bottom well of the companion plate also increased HUVEC migration as compared to the HUVEC incubated with BSA control and therefore rhTFF3 acted as stimulant of migration (Figure 48A). Exogenous rhTFF3 acted as both a chemoattractant and stimulant to induce migratory behavior of HUVEC and to promote HUVEC migration. Notably, the chemoattractant effect of rhTFF3 on the HUVEC migration is more significant than the stimulant effect of rhTFF3. Subsequently, the effect of different

concentrations of rhTFF3 (0.1, 1.0, 2.5, 5.0, 10.0 ng/mL of rhTFF3) on HUVEC migration and invasion was determined. HUVEC were plated in the membrane of the transwell insert and incubated with different concentrations of rhTFF3 or BSA control in the bottom well of the companion plate. As compared to HUVEC incubated with BSA control, exogenous rhTFF3 significantly increased HUVEC migration from 45% to 104% at varying concentrations of rhTFF3 (2.5, 5.0, 10.0 ng/mL of rhTFF3), with the maximal HUVEC migration observed at 5.0 ng/mL of rhTFF3 (Figure 48B). Additionally, exogenous rhTFF3 stimulation significantly increased HUVEC invasion from 47% to 52% at varying concentrations of rhTFF3 (2.5, 5.0, 10.0 ng/mL of rhTFF3), with maximal HUVEC invasion at 5.0 ng/mL of rhTFF3 as compared with HUVEC incubated with BSA control (Figure 48C). These observations indicated that exogenous rhTFF3 promoted HUVEC migration and invasion in a concentration-dependent manner, with maximal stimulation at 5.0 ng/mL rhTFF3.

To determine the effect of exogenous rhTFF3 stimulation on HUVEC tubule formation *in vitro*, HUVEC plated in the Matrigel were treated with different concentrations of rhTFF3 (0.1, 1.0, 2.5, 5.0, 10.0 ng/mL of rhTFF3). Exogenous rhTFF3 stimulation significantly increased tubule length formed by HUVEC from 48% to 69% at varying concentration of rhTFF3 (1.0, 2.5, 5.0 ng/mL of rhTFF3), with maximal HUVEC tubule formation *in vitro* at 5.0 ng/mL (Figure 48D-E). Higher concentration of rhTFF3 (10.0 ng/mL) exhibited a slight increase of tubule length formed by HUVEC (22%) when compared with HUVEC treated with control BSA. These observations suggested that exogenous rhTFF3 stimulation significantly increased HUVEC tubule formation *in vitro* with maximal stimulation at 5.0 ng/mL of rhTFF3 and exhibited lesser stimulatory effect on HUVEC tubule formation *in vitro* at a higher concentration of exogenous rhTFF3.

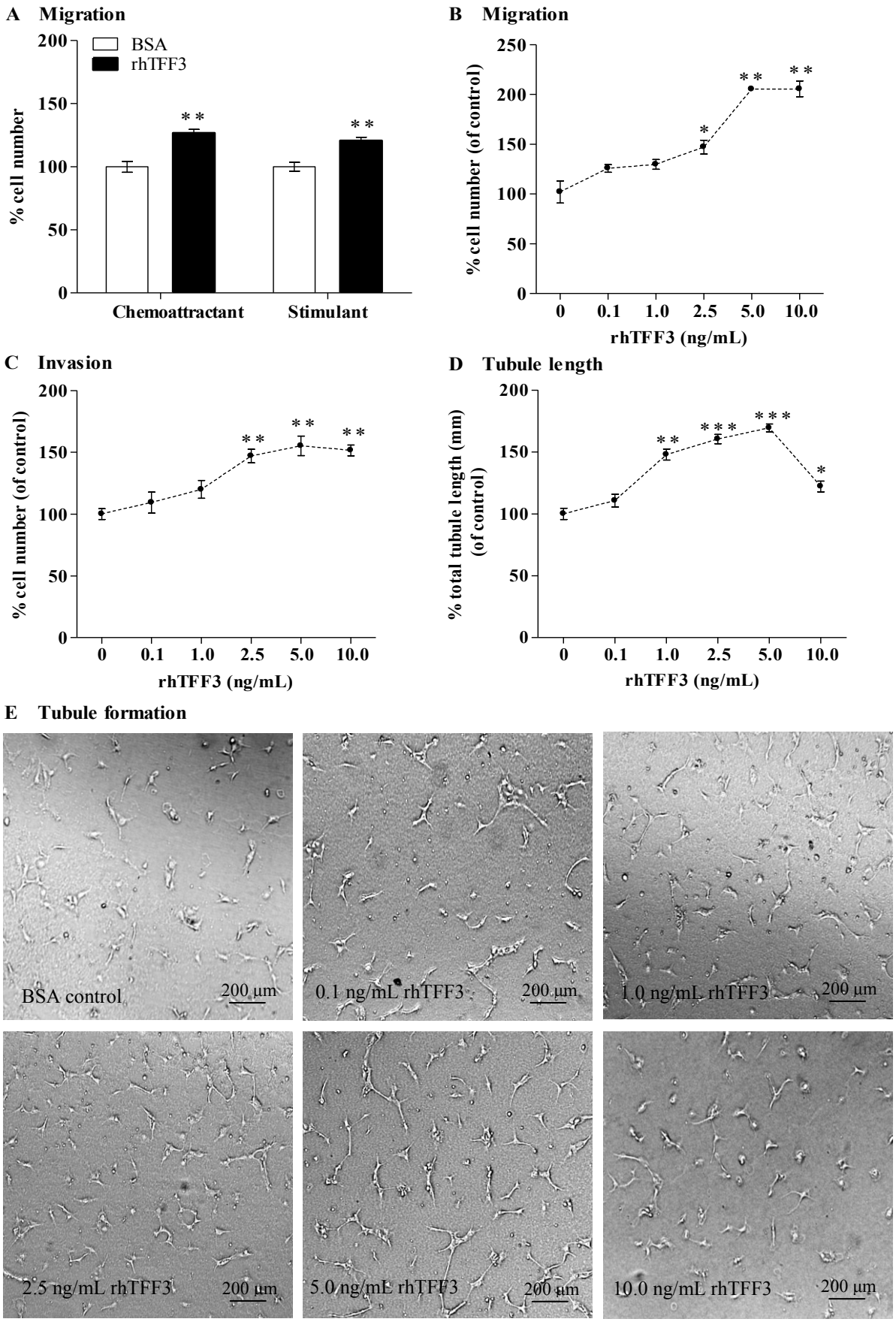

A Migration
BSA
rhTFF3
% cell number
Chemoattractant
Stimulant
B Migration
% cell number (of control)
rhTFF3 (ng/mL)
C Invasion
% cell number (of control)
rhTFF3 (ng/mL)
D Tubule length
% total tubule length (mm) (of control)
rhTFF3 (ng/mL)
E Tubule formation
BSA control
0.1 ng/mL rhTFF3
1.0 ng/mL rhTFF3
2.5 ng/mL rhTFF3
5.0 ng/mL rhTFF3
10.0 ng/mL rhTFF3
200 μm

Figure 48: Exogenous rhTFF3 stimulation increased HUVEC migration, invasion, and tubule formation *in vitro*. A, Exogenous rhTFF3 acted as chemoattractant and stimulant on HUVEC migration. For rhTFF3 to act as a chemoattractant, HUVEC were plated on the membrane of a transwell insert and incubated with either exogenous rhTFF3 (5 ng/mL) or BSA used as a control (5 ng/mL) in the bottom well of companion plate for 24 hour. For rhTFF3 to act as a stimulant, 5 ng/mL rhTFF3 or BSA control was added to the HUVEC were plated on the membrane of a transwell insert and incubated with 10% FBS EBM medium in bottom well of companion plate of for 24 hour. B, Migration of HUVEC after 24 hour incubation with different concentration of exogenous rhTFF3 (0.1, 1.0, 2.5, 5, 10 ng/mL of rhTFF3) or BSA control (10 ng/mL). C, Invasion of HUVEC after 24 hours incubation with different concentration of exogenous rhTFF3 (0.1, 1.0, 2.5, 5, 10 ng/mL of rhTFF3) or BSA control (10 ng/mL). D, Exogenous rhTFF3 stimulation in the HUVEC tubule formation *in vitro*. HUVEC plated in the Matrigel were treated with different concentration of exogenous rhTFF3 (0.1, 1.0, 2.5, 5, 10 ng/mL of rhTFF3) or BSA control (10 ng/mL). Total tubule length was assessed after 12 hour using ImageJ analysis software. E, representative light microscopy images of HUVEC tubule formation *in vitro* in which HUVEC treated with different concentrations of rhTFF3 or BSA control. BSA was used as control. HUVEC treated with BSA control was used as baseline. The light microscopy images were taken at ×40. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with BSA control; scale bar, 200 μm.

4.2.3 Forced expression of TFF3 in HUVEC increased TFF3 expression

I have demonstrated that exogenous rhTFF3 stimulation directly acted on HUVEC to promote angiogenic behaviors *in vitro*. I then determined whether TFF3 secreted from HUVEC exerted similar functional activity as exogenous rhTFF3 to promote the angiogenic behaviors of endothelial cells. HUVEC were transiently transfected with an expression vector containing TFF3 cDNA (designated as HUVEC-TFF3) or pIRESneo3 empty vector (designated as HUVEC-Vec). Forced expression of TFF3 in HUVEC increased TFF3 mRNA as compared with control HUVEC-Vec after 24 hours transfection and the expression of TFF3 mRNA in HUVEC, though slightly decreased as compared to the 24 hour of post-trnasfection, was maintained until 72 hours of post-transfection (Figure 49A). Nevertheless, HUVEC with forced expression of TFF3 exhibited higher TFF3 mRNA than the control HUVEC-Vec from 24 hours to 72 hours post-transfection. Parental HUVEC endogenously expressed undetectable or very low level of TFF3 mRNA (Figure 49B) and protein (Figure 49C) as compared to the control (MCF-7 cells). HUVEC endogenously secreted TFF3 protein to the medium and was detected by rabbit anti-TFF3 polyclonal antibody in Western blot analysis (Figure 49D). Furthermore, forced expression of TFF3 in HUVEC increased TFF3

mRNA (Figure 49E) as compared with control HUVEC-Vec. Concordantly, forced expression of TFF3 in HUVEC also increased TFF3 protein in cell lysate and secreted to the medium (Figure 49F).

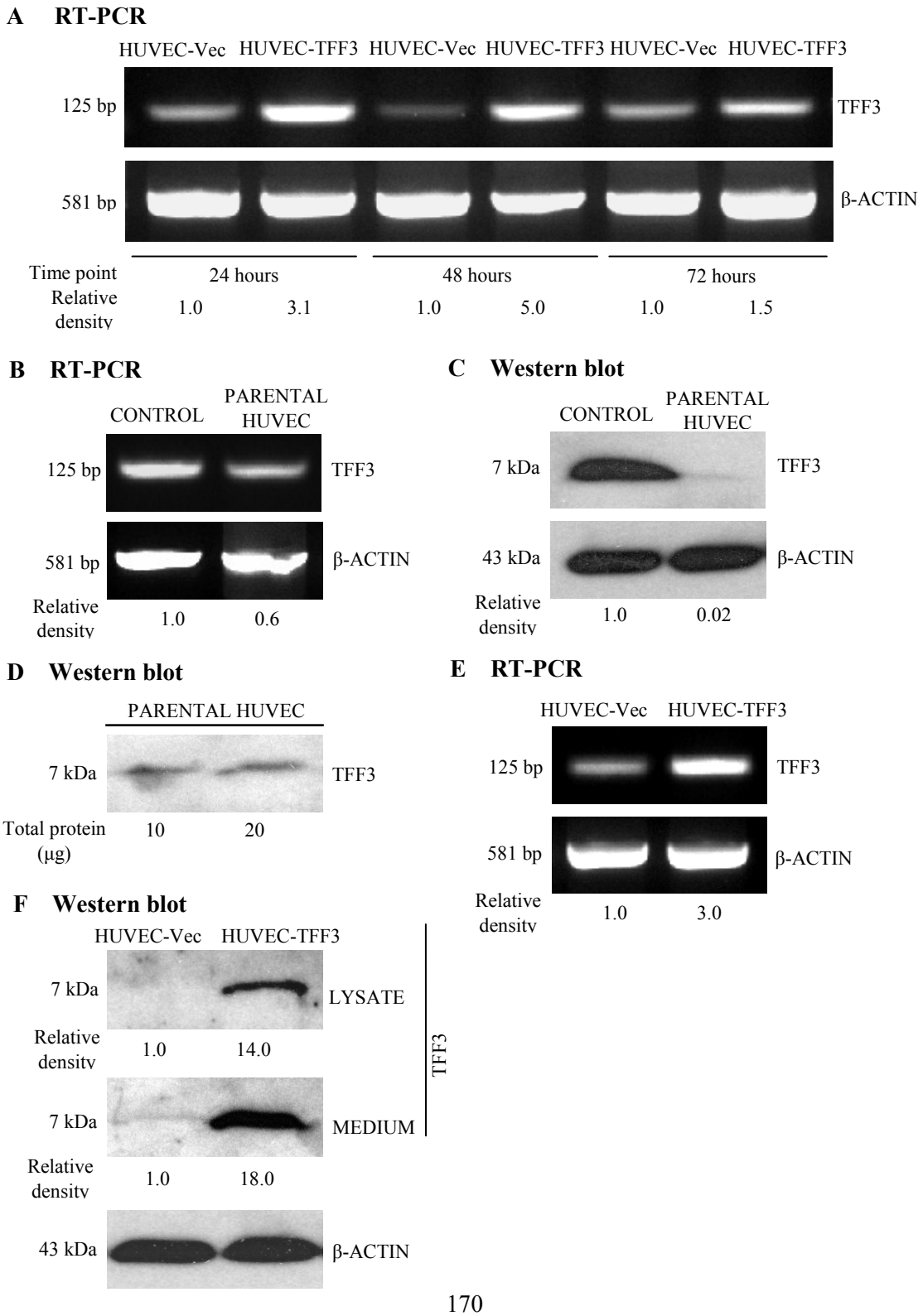


Figure 49: Forced expression of TFF3 in HUVEC increased TFF3 expression. A, Semi-quantitative RT-PCR analysis of TFF3 mRNA in HUVEC with forced expression of TFF3 after 24, 48, and 72 hours transfection. HUVEC with empty vector was used as control. B, Semi-quantitative RT-PCR analysis of TFF3 mRNA in parental HUVEC and control (MCF-7 cells). C, Western blot analysis of TFF3 protein in the cell lysate of HUVEC and control (MCF-7 cells). D, Western blot analysis of TFF3 protein in the medium of HUVEC which contained 10, 20 μg of total protein. E, semi-quantitative RT-PCR analysis of TFF3 mRNA in HUVEC with forced expression of TFF3. F, Western blot analysis of TFF3 protein in the cell lysate and conditioned medium of HUVEC with forced expression of TFF3. β-ACTIN was used as a loading control in both semi-quantitative RT-PCR and Western blot analysis.

4.2.4 Forced expression of TFF3 in HUVEC increased monolayer cell proliferation, cell cycle progression, survival

The effect of TFF3 secreted from HUVEC with forced expression of TFF3 on monolayer cell proliferation in both 10% FBS conditions (serum replete) for 72 hours and 0.2% FBS conditions (serum deprived) for 48 hours were determined. TFF3 secreted from HUVEC significantly increased monolayer cell proliferation in both 10% FBS and 0.2 % FBS conditions when compared with the control HUVEC-Vec (Figure 50A-B). When compared with control HUVEC-Vec, TFF3 secreted from HUVEC significantly increased monolayer cell proliferation by 29% in 10% FBS condition after 72 hours (Figure 50A) and by 30% in serum deprived conditions (0.2% FBS) after 48 hours (Figure 50B). These data indicated that TFF3 secreted from HUVEC promoted HUVEC monolayer cell proliferation in both 10% FBS and 0.2% FBS conditions. Furthermore, TFF3 secreted from HUVEC increased cell cycle progression, as indicated by BrdU incorporation, in both serum-free conditions and 10% FBS conditions by 30% and 43%, respectively, when compared with control HUVEC-Vec (Figure 50C). TFF3 secreted from HUVEC also decreased apoptotic cell death, as indicated by fluorescent microscopic nuclear staining, in both serum-free and 10% FBS conditions by 37% and 39%, respectively when compared with control HUVEC-Vec (Figure 50D). These data suggested that TFF3 secreted from HUVEC promoted cell proliferation and survival.

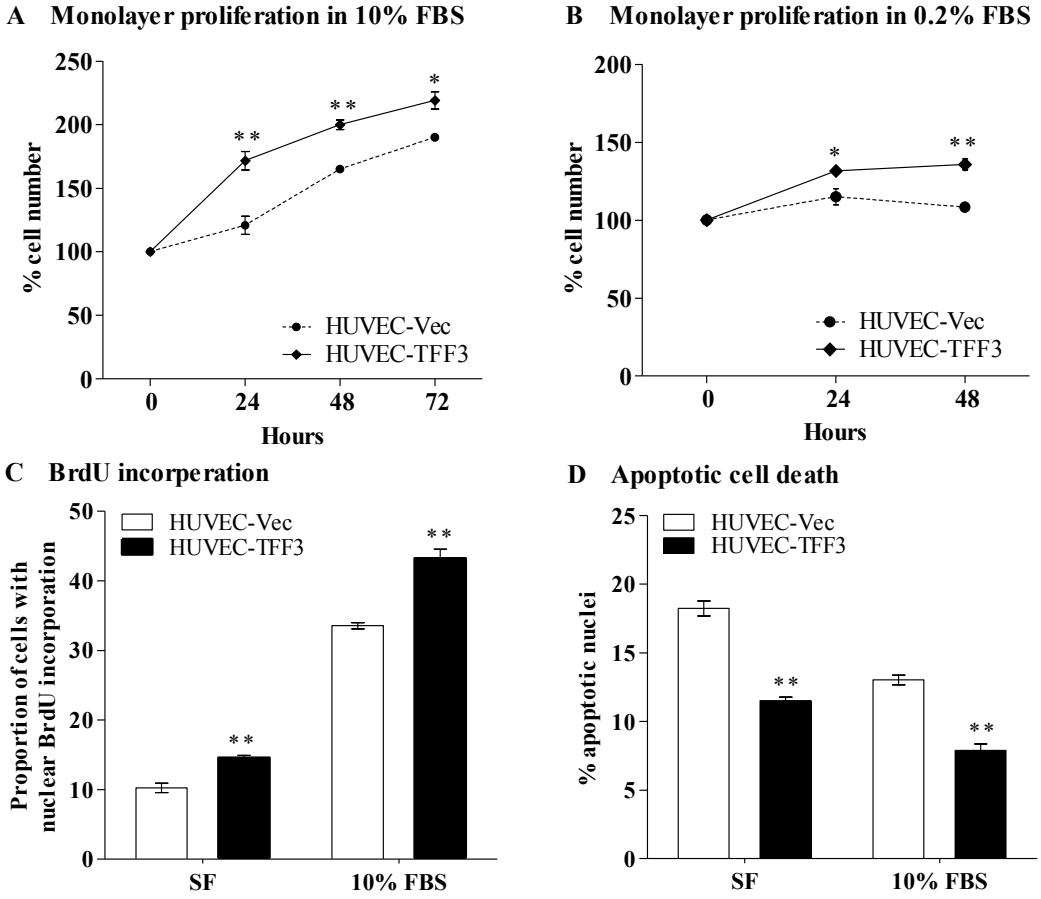


Figure 50: Forced expression of TFF3 in HUVEC increased monolayer cell proliferation, cell cycle progression, and survival. A, HUVEC monolayer cell proliferation after co-culture with HUVEC with forced expression of TFF3 in 10% FBS conditions. HUVEC with empty vector was used as control. B, HUVEC monolayer cell proliferation after co-culture with HUVEC with forced expression of TFF3 in 0.2% FBS condition. C, HUVEC cell cycle progression after 24 hours co-culture with HUVEC with forced expression of TFF3 in serum-free (SF) and 10% FBS conditions. D, HUVEC apoptotic cell death after 24 hours co-culture with HUVEC with forced expression of TFF3 in serum-free (SF) and 10% FBS conditions. *, $P < 0.05$; **, $P < 0.01$.

4.2.5 Forced expression of TFF3 in HUVEC increased migration, invasion, and tubule formation *in vitro*

To determine the effect of TFF3 secreted from HUVEC on the migratory and invasive behavior of endothelial cells, transwell migration and invasion assays were conducted. Forced expression of TFF3 in HUVEC consistently increased migration by 21% (Figure 51A) and invasion by 11% (Figure 51B) when compared with control HUVEC-Vec, suggesting that TFF3 secreted from HUVEC slightly promoted cell migration and invasion.

To determine whether TFF3 secreted from HUVEC stimulates angiogenesis *in vitro,* a tubule formation assay was conducted whereby HUVEC with forced expression of TFF3 were seeded in the Matrigel. TFF3 secreted from HUVEC significantly increased tubule length by 125% (Figure 51C) and tubule number by 56% (Figure 51D) when compared with control HUVEC-Vec. TFF3 secreted from HUVEC increased tubules formed by endothelial cells when compared with control HUVEC-Vec (Figure 51E), suggesting that TFF3 secreted from HUVEC promoted tubule formation *in vitro*.

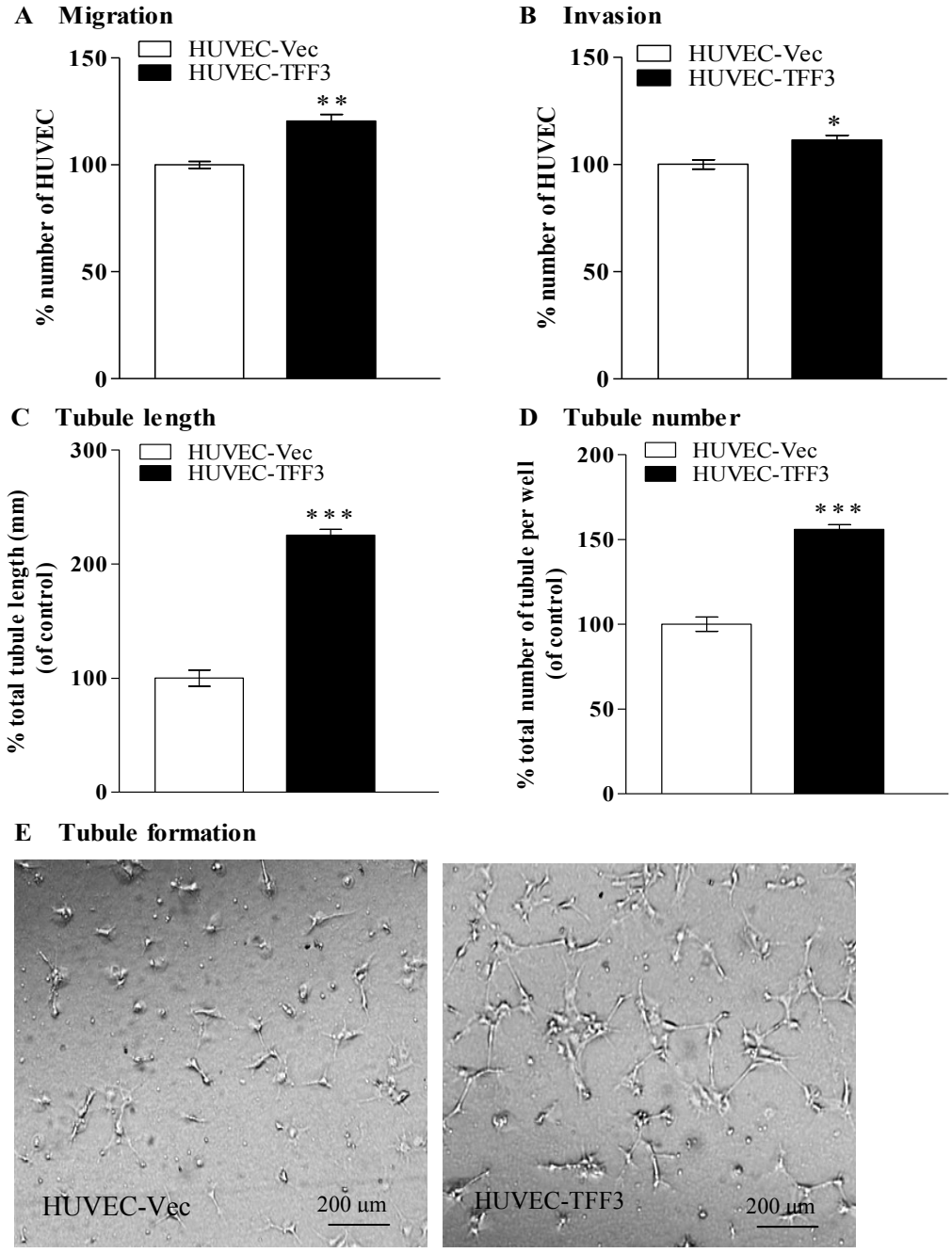


Figure 51: Forced expression of TFF3 in HUVEC increased cell migration, invasion, and tubule formation *in vitro*. A, HUVEC migration after 24 hours co-culture with HUVEC with forced expression of TFF3 in serum-free conditions. HUVEC with empty vector was used as control. B, HUVEC invasion after 24 hours co-culture with HUVEC with forced expression of TFF3 in serum-free conditions. C and D, tubule formation *in vitro* after 12 hours co-culture HUVEC with forced expression of TFF3 in the Matrigel. Total tubule length (C) and tubule number (D) were assessed using ImageJ analysis software. E, representative light microscopy images of tubule formation *in vitro* after 12 hours co-culture with HUVEC with forced expression of TFF3 in the Matrigel. The light microscopy images were taken at ×40 magnification. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; scale bar, 200 µm.

4.2.6 Depletion of TFF3 in HUVEC by siRNA decreased TFF3 expression

To determine the effect of depletion of TFF3 on the angiogenic behaviors of endothelial cells, the endogenous expression of TFF3 in HUVEC was depleted by siRNA to TFF3. HUVEC were transiently transfected with an expression vector containing TFF3 siRNA (designated as HUVEC-siTFF3) or pSilencer 2.1-U6 hygro empty vector (designated as HUVEC-siVec). Depletion of TFF3 in HUVEC decreased TFF3 mRNA and protein when compared with control HUVEC-siVec (Figure 52A-B).

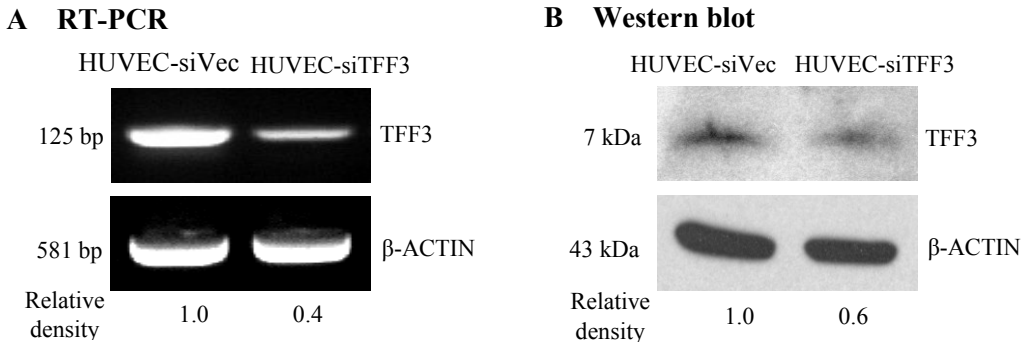


Figure 52: Depletion of TFF3 in HUVEC by siRNA decreased TFF3 expression. A, RT-PCR analysis of TFF3 mRNA in HUVEC with depletion of TFF3. HUVEC with control siRNA was used as control. B, Western blot analysis of TFF3 protein in HUVEC with depletion of TFF3. β-ACTIN was used as a loading control in both semi-quantitative RT-PCR and Western blot analysis.

4.2.7 Depletion of TFF3 in HUVEC decreased monolayer cell proliferation, cell cycle progression, and survival

The effect of TFF3 secreted from HUVEC with depletion of TFF3 by siRNA on monolayer cell proliferation in both 10% FBS conditions for 72 hours and 0.2% FBS conditions for 48 hours were determined. Depletion of TFF3 in HUVEC significantly decreased monolayer cell proliferation in both 10% FBS and 0.2% FBS conditions when compared with the control HUVEC-Vec (Figure 53A-B). When compared with the control HUVEC-siVec, depletion of TFF3 in HUVEC significantly decreased monolayer cell proliferation by 65% in 10% FBS condition (Figure 53A) and by 20% in serum deprived conditions after 48 hours (Figure 53B). Furthermore, depletion of HUVEC decreased cell

cycle progression in both serum-free conditions and 10% FBS conditions by 38% and 30%, respectively when compared with control HUVEC-Vec (Figure 53C). Depletion of TFF3 in HUVEC also increased apoptotic cell death in both serum-free conditions and 10% FBS conditions by 32% and 37%, respectively when compared with control HUVEC-Vec (Figure 53D). Depletion of TFF3 by siRNA in HUVEC decreased cell proliferation and survival.

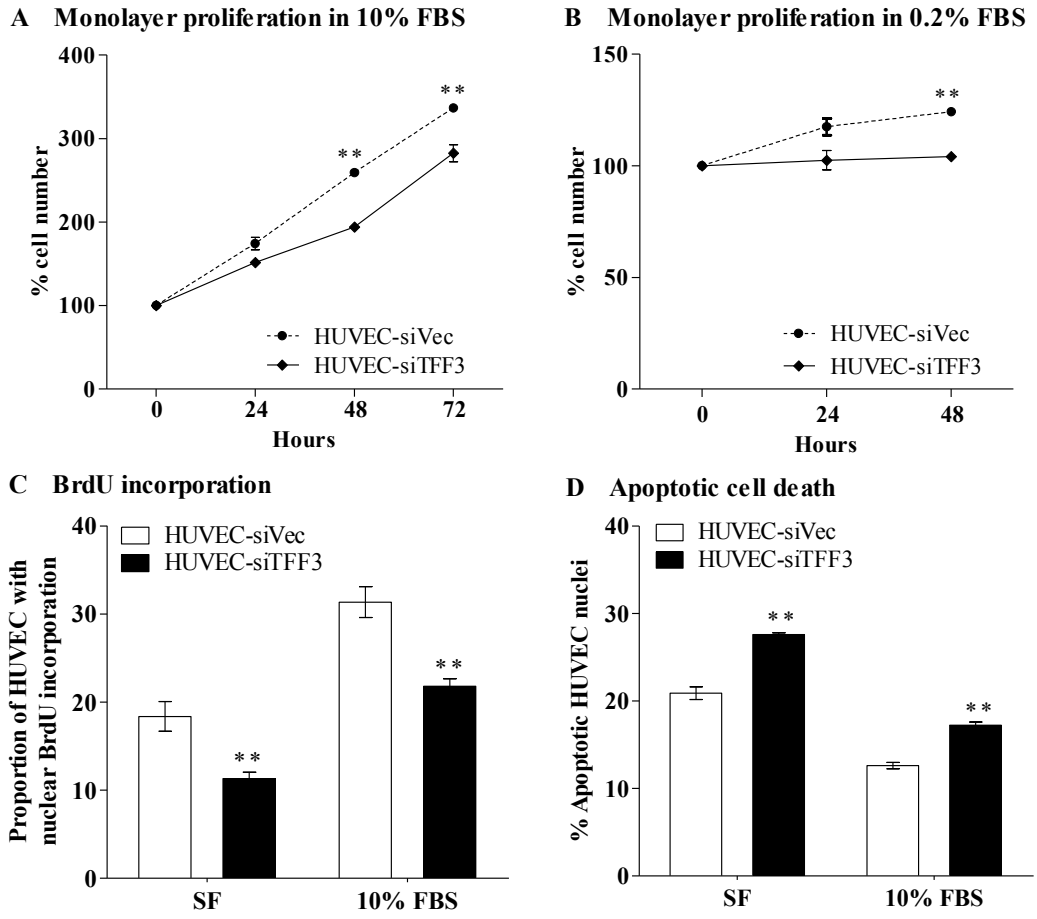


Figure 53: Depletion of TFF3 in HUVEC decreased monolayer cell proliferation, cell cycle progression, and survival. A, HUVEC monolayer cell proliferation after co-culture with HUVEC with depletion of TFF3 in 10% FBS conditions. HUVEC with control siRNA was used as control. B, HUVEC monolayer cell proliferation after co-culture with HUVEC with depletion of TFF3 in 0.2% FBS conditions. C, HUVEC cell cycle progression after co-culture with HUVEC with depletion of TFF3 in both serum-free (SF) and 10% FBS conditions. D, HUVEC apoptotic cell death after 24 hours co-culture with HUVEC with depletion of TFF3 in serum-free (SF) and 10% FBS conditions. *, $P < 0.05$; **, $P < 0.01$.

4.2.8 Depletion of TFF3 in HUVEC decreased migration, invasion, and tubule formation *in vitro*

The effect of depletion of TFF3 by siRNA on the angiogenic behaviors of HUVEC was determined. Depletion of TFF3 in HUVEC significantly decreased HUVEC migration by 37% (Figure 54A) and invasion by 28% (Figure 54B) when compared with control HUVEC-siVec. Furthermore, depletion of TFF3 by siRNA in HUVEC significantly decreased tubule length by 37% and tubule number by 38% when compared with control HUVEC-siVec (Figure 54C-D). Depletion of TFF3 in HUVEC decreased tubules formed by endothelial cells when compared with the control HUVEC-siVec (Figure 54E). Therefore, depletion of TFF3 by siRNA in HUVEC decreased angiogenic behaviors of endothelial cells.

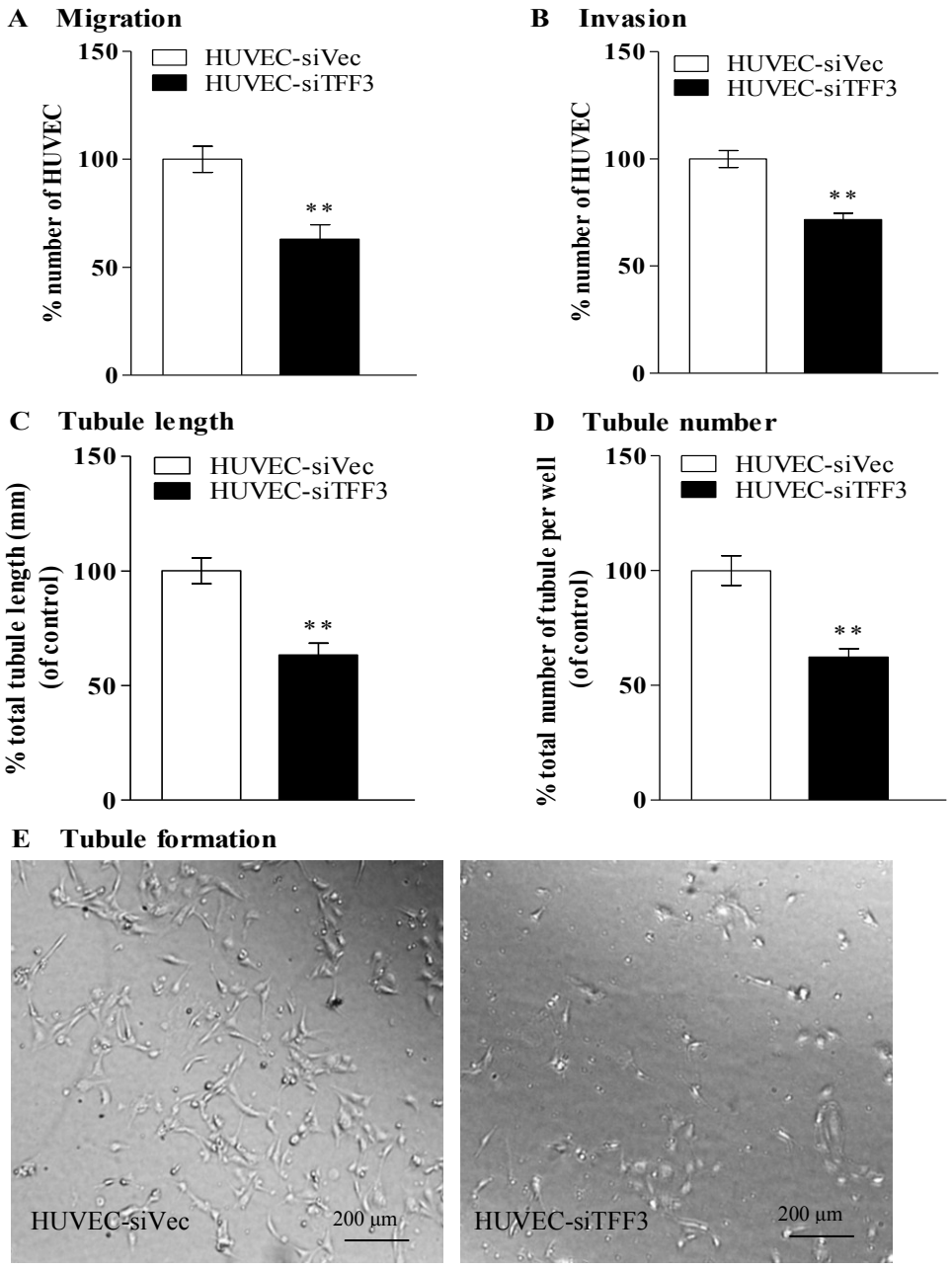


Figure 54: Depletion of TFF3 in HUVEC decreased migration, invasion, and tubule formation *in vitro*. A, cell migration after 24 hours co-culture with HUVEC with depletion of TFF3 in serum-free conditions. HUVEC with control siRNA was used as control B, cell invasion after 24 hours co-culture with HUVEC with depletion of TFF3 in serum-free conditions. C and D, tubule formation *in vitro* on Matrigel after 12 hours co-culture with HUVEC with depletion of TFF3 in serum-free conditions. Total tubule length (C) and tubule number (D) were assessed after 12 hours incubation using ImageJ analysis software. E, representative light microscopy images of tubule formation *in vitro* in the Matrigel after 12 hours co-culture with HUVEC with depletion of TFF3. The light microscopy images were taken at ×40 magnification. **, $P < 0.01$; scale bar, 200 µm.

4.2.9 Forced expression of TFF3 in HUVEC increased IL-8 promoter activity

To determine if TFF3 secreted from HUVEC regulated IL-8 promoter activity, HUVEC with forced expression of TFF3 were transiently transfected with an full length IL-8 promoter reporter vector (-4800 to +104 bp) and a pRL-CMV control reporter. Forced expression of TFF3 in HUVEC significantly increased IL-8 promoter activity (1.5-fold increase) as compared to the control HUVEC-Vec, indicating that TFF3 secreted from HUVEC stimulated IL-8 promoter activity, albeit slightly (Figure 55A). Additionally, HUVEC with forced expression of TFF3 transiently transfected with a shorter promoter, pGL-IL8-152 reporter vector (-152 to + 44 bp), significantly increased IL-8 promoter activity (2.0-fold increase) when compared with the control HUVEC-Vec (Figure 55B).

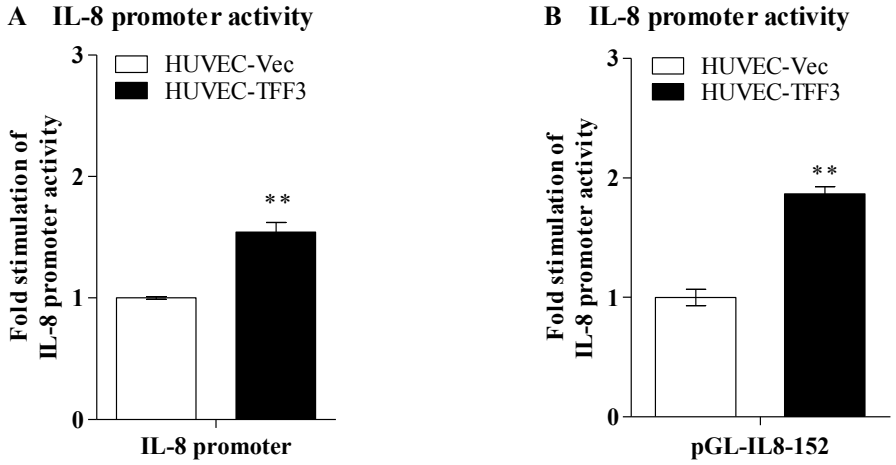


Figure 55: Forced expression of TFF3 in HUVEC increased IL-8 promoter activity. A, IL-8 promoter activity in HUVEC with forced expression of TFF3 transiently transfected with IL-8 promoter (full length, -4800 to + 104 bp). HUVEC with empty vector was used as control. B, IL-8 promoter activity in HUVEC with forced expression of TFF3 transiently transfected with pGL-IL8-152 reporter (-152 to +44 bp). Fragment containing 5' flanking regions of the IL-8 gene were subcloned upstream of a luciferase reporter gene in the pGL3-basic vector. pGL-IL8-152 reporter vector encompassing nucleotides -152 to +44 of the IL-8 promoter, which contain the transcriptional factors, AP-1, NF-IL6 and NF-κB response site. The TATA box, and the NF-IL6, NF-κB and AP-1 binding sites are located at -13, -70, -80 and -120, respectively. HUVEC with forced expression of TFF3 were transiently transfected with a reporter and a pRL-CMV control reporter. IL-8 promoter activity measured by firefly luciferase activity that normalized by *Renilla* luciferase activity. **, $P < 0.01$.

4.2.10 Forced expression of TFF3 in HUVEC increased IL-8 expression

Forced expression of TFF3 in HUVEC stimulated angiogenic behaviors of HUVEC to promote angiogenesis *in vitro*. Presumably, TFF3 modulated the expression of one or more specific angiogenic factors that can mediate the angiogenic action of TFF3, in addition to the direct action of TFF3 on the HUVEC as previously demonstrated by exogenous rhTFF3 stimulation. Forced expression of TFF3 in HUVEC exhibited relatively similar expression levels of VEGF-A (VEGF-A_{121} and VEGF-A_{165} mRNAs) and its tyrosine kinase receptors (VEGFR1 and VEGFR2 mRNAs) when compared with the control HUVEC-Vec. Forced expression of TFF3 in HUVEC regulated neither the expression of VEGF-A_{121} and VEGF-A_{165} nor its tyrosine kinase receptors VEGFR1 and VEGFR2 (Figure 56A). The mRNA expressions of VEGF-A and VEGFR1/2 were observed in HUVEC. Nevertheless, forced expression of TFF3 in HUVEC increased IL-8 mRNA (Figure 56B) and protein (Figure 56C) when compared with the control HUVEC-Vec. Forced expression of TFF3 in HUVEC slightly increased IL-8 protein secreted to medium as compared with the control HUVEC-Vec (Figure 56D).

The angiogenic function of IL-8 is activated through the binding of IL-8 to its cognate receptors, termed as CXCR1 and CXCR2, which belong to the G protein–coupled receptor family (Holmes *et al.*, 1991). HUVEC with forced expression of TFF3 expressed both CXCR1 and CXCR2 mRNAs (Figure 56E). Forced expression of TFF3 in HUVEC slightly increased IL-8 protein secreted to the medium (Figure 56D). Parental HUVEC endogenously expressed IL-8 protein in cell lysate and also secreted to the medium (Figure 57A). Additionally, exogenous rhTFF3 (at the lower concentration of rhTFF3 e.g., 0.1 and 1.0 ng/mL rhTFF3) marginally increased the expression of IL-8 mRNA (Figure 57B) and protein (Figure 57C) in parental HUVEC, suggesting that the lower concentration of exogenous rhTFF3 was able to stimulate IL-8 protein expression in parental HUVEC, albeit slightly.

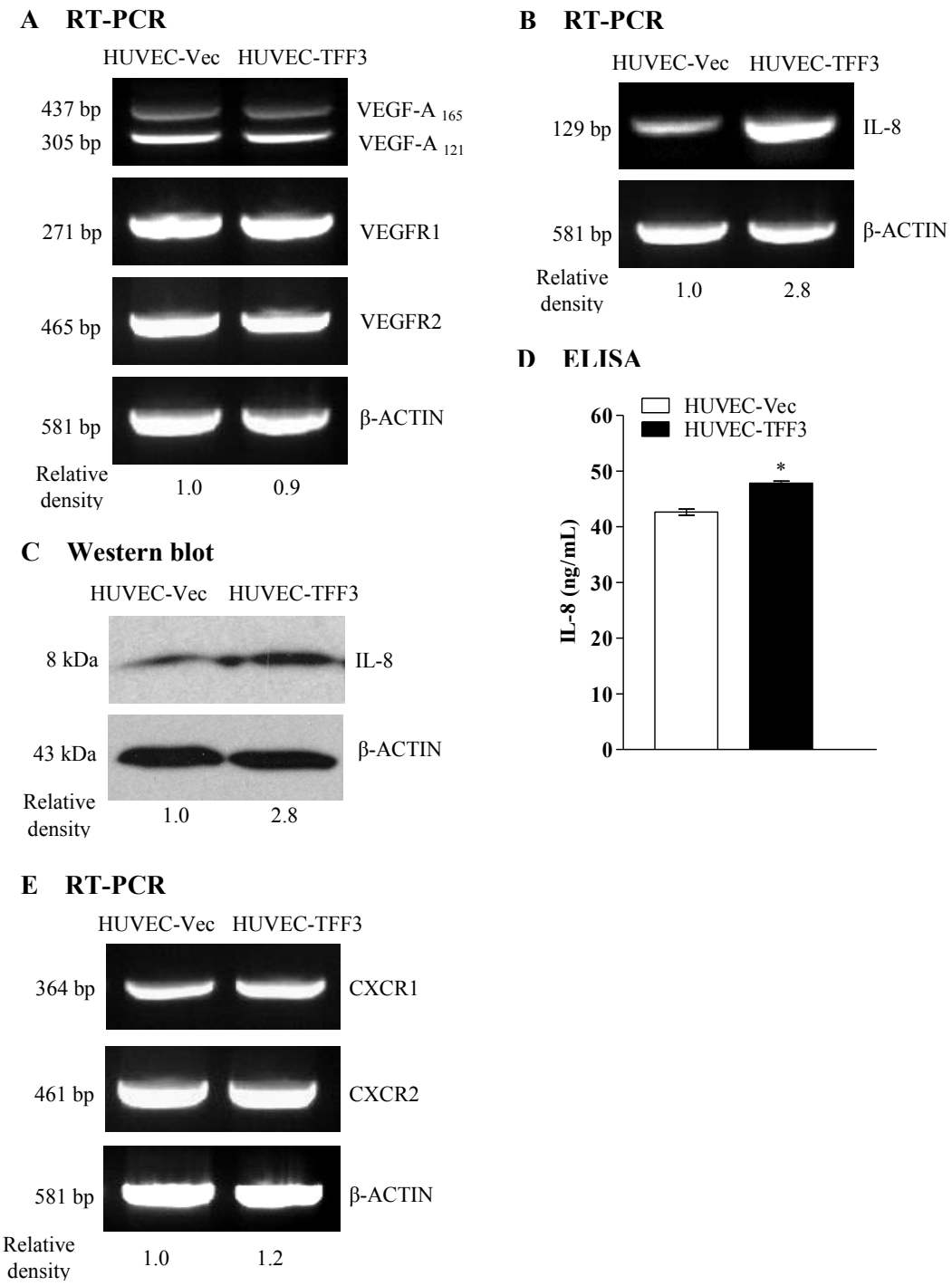


Figure 56: Forced expression of TFF3 in HUVEC increased IL-8 expression. A, semi-quantitative RT-PCR analysis of the expression of VEGF-A, VEGFR1, and VEGFR2 mRNA in HUVEC with forced expression of TFF3. HUVEC with empty vector was used as control B, semi-quantitative RT-PCR analysis of IL-8 mRNA in HUVEC with forced expression of TFF3. C, Western blot analysis of IL-8 protein in HUVEC with forced expression of TFF3. D, ELISA analysis of IL-8 protein secreted to the medium by HUVEC with forced expression of TFF3. E, semi-quantitative RT-PCR analysis of the expression of IL-8 receptors, CXCR1 and CXCR2 mRNA in HUVEC with forced expression of TFF3. β-ACTIN was used as a loading control in semi-quantitative RT-PCR and Western blot analysis. *, $P < 0.05$.

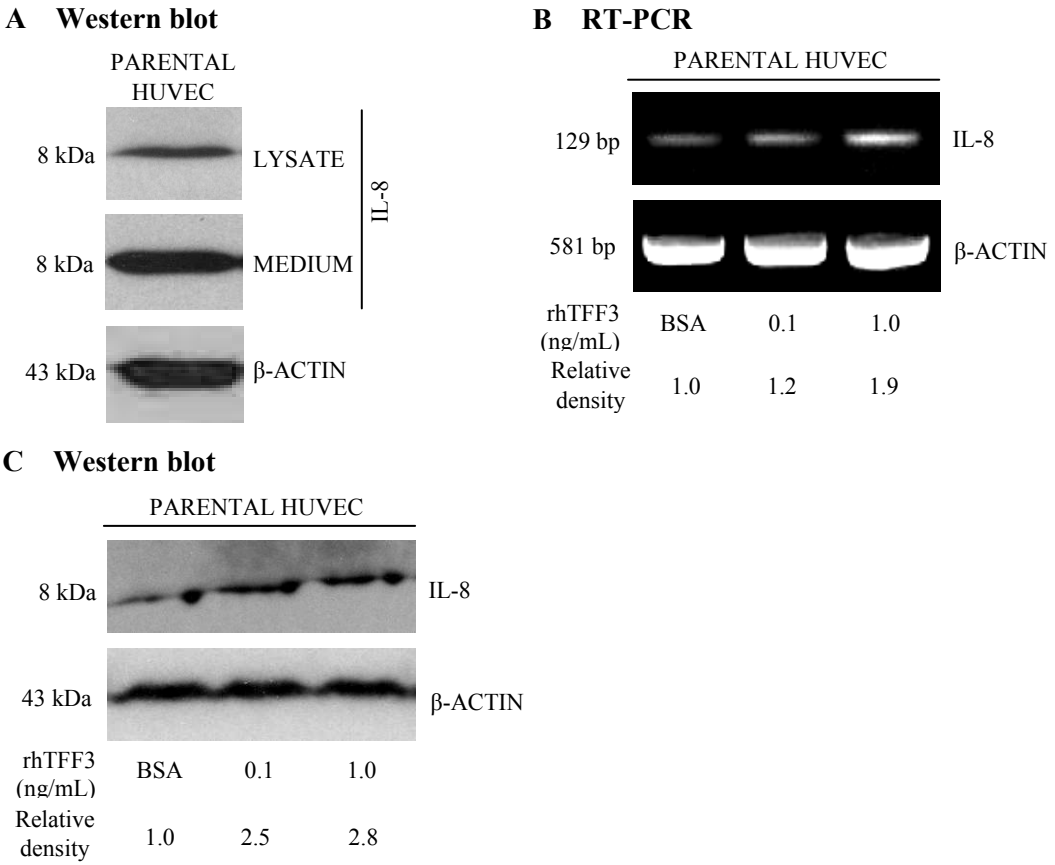


Figure 57: Exogenous rhTFF3 stimulation induced IL-8 expression in parental HUVEC. A, Western blot analysis of IL-8 protein in cell lysate and conditioned medium of Parental HUVEC. B, semi-quantitative RT-PCR analysis the expression of IL-8 mRNA in parental HUVEC treated with exogenous rhTFF3 (0.1, 1.0 ng/mL of rhTFF3) and BSA control. C, Western blot analysis of the expression of IL-8 protein in parental HUVEC treated with exogenous rhTFF3 (0.1, 1.0 ng/mL of rhTFF3) and BSA control. Parental HUVEC treated with BSA control was used as baseline. β-ACTIN was used as a loading control in semi-quantitative RT-PCR and Western blot analysis.

4.2.11 Depletion of TFF3 in HUVEC decreased IL-8 promoter activity and subsequently decreased IL-8 expression

I then determined if depletion of TFF3 in HUVEC decreased IL-8 promoter activity and subsequently its expression. Firstly, HUVEC with depletion of TFF3 was transiently transfected with full length of IL-8 promoter (-4800 to +104 bp). Depletion of TFF3 in HUVEC significantly lessened IL-8 promoter activity (2.3-fold decrease) when compared with the control HUVEC-siVec (Figure 58A). Secondly, HUVEC with depletion of TFF3 was transiently transfected with shorter IL-8 promoter, pGL-IL8-152 (-152 to + 44 bp). HUVEC

with depletion of TFF3 also lessened IL-8 promoter activity (1.9-fold decrease) when compared with the control HUVEC-siVec (Figure 58B). The transcription factors namely NF-IL-6, NF-κB, and AP-1 present at the proximal region of IL-8 promoter are required for promoting of TFF3-stimulated IL-8 promoter activity in HUVEC. Furthermore, depletion of TFF3 in HUVEC by siRNA decreased IL-8 mRNA (Figure 58C) and IL-8 protein secreted to medium (Figure 58D) when compared with the control HUVEC-siVec. Hence, depletion of TFF3 by siRNA in HUVEC lessened the IL-8 promoter activity and subsequently decreased IL-8 expression in HUVEC.

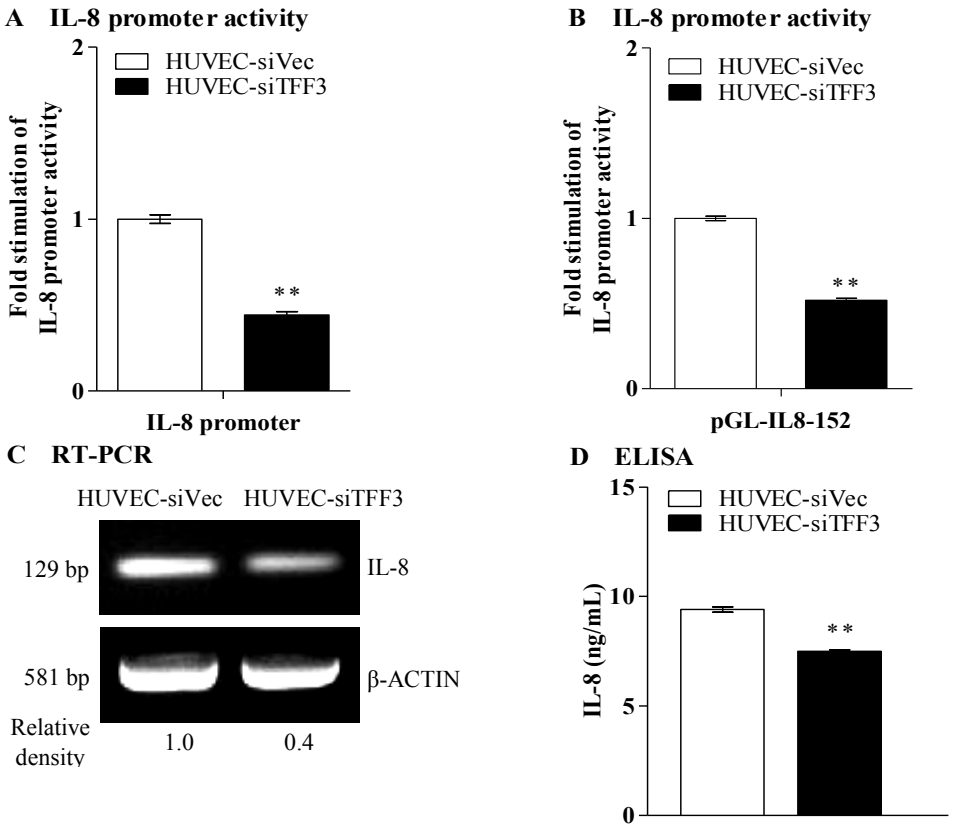


Figure 58: Depletion of TFF3 in HUVEC decreased IL-8 promoter activity and subsequently decreased IL-8 expression. A, IL-8 promoter activity in HUVEC with depletion of TFF3 transiently transfected with IL-8 promoter (full length, -4800 to + 104 bp). HUVEC with control siRNA was used as control. B, IL-8 promoter activity in HUVEC with depletion of TFF3 transiently transfected with pGL-IL8-152 reporter (-152 to +44 bp). HUVEC was transiently transfected with a reporter and a pRL-CMV control reporter. C, semi-quantitative RT-PCR analysis of IL-8 mRNA in HUVEC with depletion of TFF3. D, ELISA analysis of IL-8 protein secreted to the medium by HUVEC with depletion of TFF3. β-ACTIN was used as a loading control in semi-quantitative RT-PCR. **, $P < 0.01$.

4.3 Discussion

4.3.1 Exogenous rhTFF3 stimulation promoted angiogenic activity of HUVEC

Herein, I have demonstrated that exogenous rhTFF3 promoted HUVEC monolayer proliferation, migration, invasion, tubule formation *in vitro* in a concentration-dependent manner. Exogenous rhTFF3 directly acted on HUVEC to stimulate angiogenesis *in vitro*. Exogenous rhTFF3 stimulation increased HUVEC monolayer proliferation in 10% FBS conditions and induced a small increase of HUVEC monolayer proliferation in serum deprived conditions (0.2% FBS). Hence, exogenous rhTFF3 was able to stimulate HUVEC monolayer proliferation in both 10% FBS and serum deprived conditions. These results are supported by Kindon *et al.* (1995) who previously demonstrated that recombinant TFF3 protects human colon cancer cell monolayer from external insults. Protection of colon cancer cell from injury or damage was enhanced by the addition of both recombinant TFF3 and human colonic mucin (Kindon *et al.*, 1995). We have identified that TFF3 acted as mitogenic and cytoprotective factor.

All TFF peptides have been demonstrated to be motogenic and are involved in wound healing process by promotion of cell migration and resistance to anoikis. TFF3 and TFF1 are potent motogens in both normal and cancer cells. These peptides are able to promote migration of epithelial cells, TFF3 is the only family member that has been reported to be a crucial factor implicated in mucosa restitution (Mashimo *et al.*, 1996, Poulsom, 1996). Earlier studies have reported that exogenous rhTFF3 stimulated migration of intestinal epithelial cells *in vitro* (Dignass *et al.*, 1994, Chinery and Playford, 1995). Additionally, Taupin *et al.* (1999) showed that the migration rate of epidermal cells was significantly increased after rhTFF3 treatment. Furthermore, Li *et al.* (2011) recently demonstrated that rhTFF3 slightly increased the expression of EGF, enhanced the restitution of the damaged mucosa and increased migration of the epithelial cells, thus promoting the healing of injured mucosa in colitis (Li *et al.*, 2011). Concordantly, I have demonstrated that exogenous rhTFF3 may function as both chemoattractant and stimulant to promote migration of endothelial cells but more remarkable

as a chemoattractant. I suggested that TFF3 is a potent chemotactic factor for endothelial cells and stimulates directional movement of endothelial cells towards increasing concentration of rhTFF3. I have also shown that exogenous rhTFF3 promoted HUVEC migration and invasion in a concentration-dependent manner with maximal stimulation at 5.0 ng/mL rhTFF3. Exogenous rhTFF3 is able to stimulate HUVEC migration and invasion. Notably, the effect of exogenous rhTFF3 on HUVEC migration was more prominent than invasion. Similarly, exogenous rhTFF1 stimulates migration and proliferation of mammary carcinoma cells (May and Westley, 1997a, Prest *et al.*, 2002). Prest *et al*. (2002) has suggested that rhTFF1 may stimulate cell migration by interacting with cell surface receptors. After mucosal injury, a functional EGF receptor is required for activation of TFF gene transcription through the Ras/MEK/MAP kinase signaling pathway (Taupin *et al.*, 1999). Since activation of MAP kinase enhanced cell migration, the effects of trefoil peptides on epithelial restitution may occur through MAP kinase signaling pathway (Taupin *et al.*, 1999). TFF peptides indirectly activated EGF-R to stimulate the TFF-mediated signaling events (McKenzie *et al.*, 1997, Taupin *et al.*, 1999). TFF peptides and EGF have been reported to exert synergistic effects in promotion of cell migration (Poulsom, 1996). However, TFF3 stimulates cell invasion through an EGFR-independent signaling pathway (Rodrigues *et al.*, 2003b). TFF3 induced intestinal epithelial cells resistance to apoptosis via activation of nuclear factor-κB (NF-κB), a pivotal regulator implicated in survival pathway of intestinal epithelial cells. Hence, intestinal epithelial cells treated with recombinant TFF3 were resistant to anoikis (Chen *et al.*, 2000). The anti-apoptotic function of TFF3 in colon cancer was regulated via an alternative signaling cascade such as PI3K, protein kinase B (PKB), and NF-κB. Blocking of EGF signaling abrogated TFF3-stimulated cell survival and partially inhibited Erk/MAPK-dependent transcriptional signaling following TFF3 stimulation (Taupin *et al.*, 1999). These observations suggested that TFF peptides stimulated activation of Erk/MAPK through alternative pathways.

Addition of exogenous recombinant human TFF1 and TFF3 has been reported to promote formation of capillary vessel in a CAM assay. Exogenous rhTFF1 stimulated

HUVEC to form tubules in the Matrigel and the angiogenic activity of rhTFF1 is comparable to that stimulated by VEGF-A and TGF-α (Rodrigues *et al.*, 2003b). Since TFF3 is analogous with TFF1, TFF3 may also function as pro-angiogenic factor to promote angiogenesis *in vivo*. Concordant with these observations, I have demonstrated that exogenous rhTFF3 directly acted on endothelial cells and promoted tubule formation *in vitro* in a concentration-dependent manner with the maximal stimulation at 5.0 ng/mL rhTFF3. Notably, higher concentrations of exogenous rhTFF3 (10 ng/mL rhTFF3) exerted lesser stimulatory effect on HUVEC tubule formation *in vitro*. Previous reports have demonstrated that 100 nM of recombinant TFF1 and TFF3 were needed to stimulate capillary vessel formation in CAM assay or tubule formation *in vitro* (Rodrigues *et al.*, 2003b). Additionally, migration of MCF-7 cell lines was stimulated by 10 μg/mL of recombinant TFF1 (Prest *et al.*, 2002). In relation to this observation, Prest *et al.*, (2002) proposed that TFF1 and possibly TFF3, at the higher concentrations may inhibit dimerization of the putative TFF receptor and subsequently lead to attenuation of receptor activation for signal transduction. Angiogenesis *in vitro* induced by TFF peptides was demonstrated to be dependent on both cyclooxegenase (COX-2) and the EGF-R tyrosine kinase to mediate pro-angiogenic activity of TFF3 (Rodrigues *et al.*, 2001). Increased expression of COX-2 promotes resistance to apoptosis and tumor angiogenesis as well as enhances invasion and metastasis (Labayle *et al.*, 1991, Jacoby *et al.*, 1996, Tsujii *et al.*, 1998). Furthermore, nitric oxide plays an important role in tumor angiogenesis, and TFF3 has been demonstrated to enhance nitric oxide (NO) formation through the inducible nitric oxide synthase (iNOS/NOS2) (Tan *et al.*, 1999).

Although TFF1 and TFF3 peptides only contain a single trefoil domain, they are capable of forming homodimers via utilization of an unpaired cysteine residue located near the C-terminus (May *et al.*, 2003). TFF1 dimer is more potent in stimulating migration of mammary carcinoma cells via a bivalent dimeric receptor (Marchbank *et al.*, 1998, Prest *et al.*, 2002). Additionally, TFF1 dimer is more biologically active than TFF1 monomer in preventing drug-induced gastric damage and promoting cell migration. It has been postulated

that dimerization may enhance the interaction of TFF1 with its receptor (Marchbank *et al.*, 1998). The relative biological activity of dimeric and monomeric recombinant TFF3 has been compared in a mice model of ischaemic necrotising enterocolitis. TFF3 dimer was shown to possess a similar potency as the TFF3 monomer and both forms of recombinant TFF3 promoted healing of intestinal injury induced by ischemia (Carrasco *et al.*, 2004). It has been suggested that TFF3 dimer is necessary for activation of signal transduction pathways in intestinal epithelial cells, for instance PI3K and NF-κB survival pathways (Taupin *et al.*, 1999).

The combined functions of TFF peptides and mucin may cooperatively protect the mucosa from injury and increase mucus viscosity (Kindon *et al.*, 1995). The interaction between TFF peptide and mucin has also been demonstrated to enhance cell migration and nitric oxide production (Dignass *et al.*, 1994, Tan *et al.*, 1999). TFF1 is co-localized with MUC5AC and TFF3 is co-localized with MUC2 in the gastrointestinal tract, indicating that specific TFF peptides may have specific mucin partner (Longman *et al.*, 2000). Moreover, Chinery and Cox (1995) have suggested that TFF3 binds to a phosphorylated tyrosine-containing peptide of 28 kDa. Recent evidence has reported that mutation in the brichos domain of SP-C promoted annexin V association and subsequent apoptosis by activating caspase 3 (Mulugeta *et al.*, 2005), suggesting that there was a putative binding between trefoil factors and the brichos domain, which can abrogate anti-apoptotic activity of TFF3 (Mulugeta *et al.*, 2005). Many reports pertaining to the functions of trefoil factors are suggestive of a receptor-mediated mode of action such as activation of tyrosine kinase or tyrosine phosphatase, and signaling via transduction pathways such as RAS/MEK/MAPK (Kanai *et al.*, 1998, Taupin *et al.*, 1999). However, there is still no trefoil factors binding molecule with the canonical characteristics of a receptor that has yet been identified (Otto and Thim, 2005). A recent study suggested TFF3 receptor is present in the intestinal epithelial cells and the binding between this receptor and TFF3 is as if specific receptor-ligand interaction. The

identity of this specific TFF3 receptor is yet to be identified and required for further investigation (Yong *et al.*, 2013).

In summary, exogenous rhTFF3 can directly stimulate angiogenic behaviors of endothelial cells, although HUVEC endogenously expressed relatively low level of TFF3. Hence, exogenous rhTFF3 is able to stimulate angiogenesis *in vitro*.

4.3.2 Forced expression of TFF3 in HUVEC promoted angiogenesis

I have demonstrated that forced expression of TFF3 in HUVEC promoted angiogenic behavior of endothelial cells through an autocrine loop. Additionally, TFF3 secreted from HUVEC may exhibit similar functions as exogenous rhTFF3 stimulation and thus stimulated angiogenic behaviors of endothelial cells. The effect of forced expression of TFF3 in HUVEC was generally not very drastic because the function of TFF3 in endothelial cells is more predisposed in maintaining the angiogenic activity and normal cellular processes (Kinoshita *et al.*, 2000a, Podolsky, 2000, Taupin *et al.*, 2000a). The ability of TFF3 to stimulate angiogenic behaviors of endothelial cells was consistent with others secreted protein such as Artemin secreted from mammary carcinoma cells that exhibited its stimulatory effect on HMEC-1 around 20 - 30% (Banerjee *et al.*, 2011). Endothelial cells expressed undetectable or very low level of TFF3 (Dhar *et al.*, 2005, Kjellev, 2009). Concordantly, HUVEC utilized in this study also expressed undetectable or very low level of TFF3 protein. Forced expression of TFF3 in HUVEC promoted monolayer cell proliferation, cell cycle progression, survival, migration, invasion, and tubule formation *in vitro*. Depletion of TFF3 by siRNA in HUVEC decreased angiogenic behaviors of endothelial cells. In general, increased TFF3 expression in HUVEC exhibited lesser effect in cellular processes than exogenous rhTFF3 stimulation.

The mitogenic role of TFF3 in normal and cancer cells are controversial. Over-expression of TFF3 has been reported to decrease cell proliferation of human gastrointestinal cells (Uchino *et al.*, 2000, Bossenmeyer-Pourie *et al.*, 2002). In contrast, increased TFF3

expression in mammary carcinoma cells promoted cell proliferation (Xu *et al.*, 2005, Kannan *et al.*, 2010). I have demonstrated that TFF3 secreted from HUVEC promoted monolayer cell proliferation by increased cell cycle progression and decreased apoptotic cell death, suggesting that TFF3 secreted from HUVEC may act as pro-mitogenic and pro-survival factor. Concordantly, TFF3 was shown to protect intestinal epithelial cells from apoptosis after mucosal damage is dependent on PI3K and EGF-R signaling pathways. Additionally, TFF3 also promotes serine phosphorylation of Akt kinase, which is associated with cell survival (Kinoshita *et al.*, 2000b, Podolsky, 2000, Taupin *et al.*, 2000b).

It is well established that TFF3 promotes intestinal epithelial cell migration and resistance to apoptosis in the process of mucosa restitution (Dignass *et al.*, 1994, Playford *et al.*, 1995). The pro-migratory function of TFF3 has furthermore been observed in epithelial cells not from the gastrointestinal tract, suggesting that TFF peptides are normally expressed in any type of epithelial cells and its functions are cell-type specific (Kjellev, 2009). TFF3 is an anti-apoptotic protein secreted by goblet cells to protect intestinal epithelial cells from anoikis (Regalo *et al.*, 2005). It has been shown that TFF3 stimulated intestinal epithelial cell resistance to anoikis via activation of NF-κB signaling pathway (Chen *et al.*, 2000). Another study has reported that TFF3 exerted anti-anoikis effects on the intestinal cells through activation of EGF-R signaling pathway (Kinoshita *et al.*, 2000a). Consistent with these previous reports, I have demonstrated that TFF3 secreted from HUVEC promoted cell migration, invasion and tubule formation *in vitro*, supporting that TFF3 enhanced angiogenic behaviors of HUVEC to promote angiogenesis *in vitro*. Similarly, TFF3 secreted from mammary carcinoma cells also was able to stimulate angiogenic behaviors of HUVEC and promoted *de novo* angiogenesis in mammary carcinoma via STAT3/IL-8 dependent signaling pathway.

Cancer cells often lose cadherin function in the transition to an invasive and metastatic phenotype (Perl *et al.*, 1998, Cavallaro *et al.*, 2002). Experimental evidence has suggested that TFF3 promoted cell migration requires modulation of E-cadherin function and

tyrosine phosphorylation of β-catenins (Liu *et al.*, 1997, Efstathiou *et al.*, 1998, Efstathiou *et al.*, 1999). TFF3 stimulated migration and invasion of rat fibroblastic cells that correlated with down-regulated E-cadherin and tissue inhibitor of metalloproteinase-1 (TIMP-1) as well as increased β-catenin and metalloproteinase-9 (MMP-9) (Chan *et al.*, 2005b). TFF3 has also been shown to promote colon cancer cell migration through repression of E-cadherin-catenin complex formation (Emami *et al.*, 2004). Additionally, TFF3 promoted migration of both normal and oncogenically transformed bronchial epithelial cells through activation of MAPK pathway (Graness *et al.*, 2002). Collectively, TFF3 may also promote cell migration of endothelial and mammary carcinoma cells through the same signaling pathways.

In the past decades, numerous of angiogenic factors have been reported that can promote both differentiation and proliferation of endothelial cells. Of these angiogenic factors, VEGF-A and IL-8 are the most potent regulators of tumor angiogenesis (Koch *et al.*, 1992, Nor *et al.*, 1999). VEGF-A is also known as an endothelial-cell-specific mitogen and vascular permeability factor that promotes *de novo* angiogenesis (Nor *et al.*, 1999). Nevertheless, I have observed that TFF3 secreted from HUVEC may not able to stimulate the expression of VEGF-A and its receptors, indicating that VEGF-A was probably not involved in TFF3-stimulated tumor angiogenesis. Another potent angiogenic factor, IL-8 is synthesized in multiple different cell types including cancer and endothelial cells (Polverini, 1995, Strieter *et al.*, 1995b). IL-8 binds to its cognate receptors to mediate endothelial proliferation, survival and tumor angiogenesis (Rollins, 1997, Addison et al., 2000). It has been reported that that CXCR1 and CXCR2 are expressed in endothelial cells and thus IL-8 interacts with endothelial cells through these receptors and induces tumor angiogenesis (Murdoch *et al.*, 1999, Addison *et al.*, 2000, Salcedo *et al.*, 2000b). Consistent with these reports, I have demonstrated that TFF3 secreted from HUVEC slightly increased IL-8 expression. Parental HUVEC endogenously expressed IL-8 but its expression was also marginally increased in response to exogenous TFF3 stimulation. Both CXCR1 and CXCR2 mRNAs were present in HUVEC with forced expression of TFF3.

Increased the expression of IL-8 has been characterized in cancer cells and endothelial cells. IL-8 has the capacity to exert oncogenic effects in the tumor microenvironment. For example, secretion of IL-8 can activate endothelial cells in the tumor vasculature to promote angiogenesis (Oude Nijhuis *et al.*, 2003, Ramjeesingh *et al.*, 2003, Vadeboncoeur *et al.*, 2003). Previous reports have highlighted that IL-8 promoter activity appears to be regulated by the combinatorial activation and binding of NF-κB, NF-IL-6, and AP-1 families of transcription factors (Mukaida *et al.*, 1990, Roebuck, 1999). I have observed that TFF3 secreted from HUVEC stimulated IL-8 promoter activity and subsequently lead to a slight increase of IL-8 expression. Additionally, the transcription factors namely NF-κB, NF-IL-6, and AP-1 are required for promoting TFF3-stimulated IL-8 promoter activity in HUVEC.

In summary, TFF3 secreted from endothelial cells promoted monolayer proliferation, cell cycle progression, survival, migration, invasion and tubule formation *in vitro*. Depletion of TFF3 in HUVEC diminished the ability of TFF3 on stimulation of angiogenic behaviors of endothelial cells. TFF3 secreted from HUVEC stimulated angiogenic behaviors of endothelial cells through an autocrine manner.

CHAPTER 5

General Conclusion and Future Direction

5.1 General conclusion

5.1.1 TFF3 promoted *de novo* angiogenesis in mammary carcinoma

The main focus of this study was to investigate the role of TFF3 in promoting *de novo* angiogenesis in mammary carcinoma and delineate the underlying mechanism by which TFF3 may exert this effect. Herein, I have demonstrated that TFF3 secreted either from mammary carcinoma cells or HUVEC stimulated angiogenic behaviors of endothelial cells and thus promote *de novo* angiogenesis. TFF3 secreted from mammary carcinoma cells exhibited more compelling effect on stimulation of angiogenic behaviors of endothelial cells when compared with the autonomous effect of TFF3 in HUVEC. TFF3 has been shown to promote survival, invasion, and metastatic expansion of mammary carcinoma (Kannan *et al.*, 2010, Ahmed *et al.*, 2012, Pandey *et al.*, 2014). In addition to other growth factors produced by mammary carcinoma cells (Perry *et al.*, 2008), TFF3 was able to stimulate angiogenic behaviors of endothelial cells. In contrast, the autonomous effect of TFF3 in HUVEC was generally not very drastic because the role of TFF3 in endothelial cells is more predisposed in maintaining angiogenic activity and normal cellular processes (Kinoshita *et al.*, 2000a, Podolsky, 2000, Taupin *et al.*, 2000a). It is known that the functions of TFF3 are cell-type specific, for instance, TFF3 normally expressed in gastric mucosa and is involved in protection of GI tract against mucosal damage (Taupin and Podolsky, 2003, Hoffmann, 2006), however increased TFF3 expression promotes tumorigenesis of mammary carcinoma (Kannan *et al.*, 2010, Ahmed *et al.*, 2012, Pandey *et al.*, 2014).

I have demonstrated that TFF3 secreted from mammary carcinoma cells stimulated HUVEC monolayer proliferation, cell cycle progression, survival, migration, invasion, and tubule formation *in vitro*. Depletion of TFF3 in mammary carcinoma cells by siRNA diminished HUVEC monolayer proliferation, cell cycle progression, survival, migration,

invasion and tubule formation *in vitro*. In a xenograft model, forced expression of TFF3 in MCF-7 cells produced tumors with increased IL-8 protein expression and enhanced microvessel density (as indicated by increased area of both CD31- and CD34-labeled cells) when compared with tumors produced by control MCF7-Vec cells. These observations further confirmed that TFF3 promotes tumor angiogenesis *in vivo*.

Tumor angiogenesis is modulated by a large number of secreted angiogenic proteins (Sudhakar, 2009). I therefore examined a range of angiogenic factors implicated in angiogenesis and observed that TFF3 regulated the gene expression of IL-8. Increased TFF3 expression in mammary carcinoma cells promoted IL-8 expression. The functional roles of IL-8 in promotion of invasion, survival and chemotherapeutic drug resistance of prostate cancer cell lines could be effectively inhibited by IL-8 siRNA (Singh and Lokeshwar, 2009). Hence, I employed the same IL-8 siRNA to selectively deplete IL-8 expression in MCF-7 cells with forced expression of TFF3. TFF3 secreted from mammary carcinoma cells stimulated IL-8 promoter activity and subsequently increased the expression of IL-8. Depletion of IL-8 by siRNA in mammary carcinoma cells with forced expression of TFF3 decreased IL-8 protein expression. Depletion of IL-8 by siRNA in mammary carcinoma cells significantly abrogated the stimulatory effect of TFF3 on HUVEC migration, invasion and tubule formation *in vitro*. It has been reported that neutralizing antibodies to IL-8, CXCR1 or CXCR2 abrogated endothelial cell proliferation, survival, migration, and angiogenesis as compared to control antibody (Li *et al.*, 2005). Therefore, I utilized the same clone of anti-IL-8 monoclonal antibody inhibitory to TFF3 on stimulation of angiogenic behaviors of endothelial cells mediated by IL-8. Blocking of IL-8 in mammary carcinoma cells by anti-IL-8 monoclonal antibody inhibited the ability of TFF3 on stimulation of HUVEC tubule formation *in vitro*. These results suggested that depletion of IL-8 in mammary carcinoma cells by siRNA or inhibition of IL-8 with anti-IL-8 monoclonal antibody abrogated the ability of TFF3 to stimulate the angiogenic behaviors of endothelial cells. Additionally, blocking of CXCR1 in HUVEC by anti-CXCR1 monoclonal antibody may not be able to inhibit the

stimulatory effect of TFF3 on HUVEC tubule formation *in vitro*. However, blocking of CXCR2 in HUVEC by use of anti-CXCR2 monoclonal antibody inhibited the stimulatory effect of TFF3 on HUVEC tubule formation *in vitro* mediated by IL-8. It is known that CXCR2 receptor has other ligands such as IL-1, IL-2 and IL-6 (Addison *et al.*, 2000). It is possible that anti-CXCR2 is disrupting IL-8 independent events. Hence, the ability of TFF3 to stimulate angiogenic behaviors of endothelial cells was mediated by IL-8/CXCR2 axis.

IL-8 is synthesized in different cells in response to inflammatory stimuli such as that provided by IL-1 and TNF-α (Brasier *et al.*, 1998). Up-regulation of IL-8 gene expression is achieved through activation of NF-κB in concert with other transcription factors and also dependent on cell type and stimuli (Mukaida *et al.*, 1994). IL-8 promoter contains response elements for a number of different transcription factors and has been reported to constitute with pro-inflammatory responsive elements important for IL-8 gene expression (Mukaida *et al.*, 1989, Mukaida *et al.*, 1994). Several studies have suggested that production of IL-8 protein is regulated by STAT3 through direct binding to consensus binding site at the proximal region of IL-8 promoter gene (Seidel *et al.*, 1995, Oka *et al.*, 2010).

Herein, I have demonstrated that the transcription factors namely AP-1, NF-IL-6, and NF-κB at the proximal region of IL-8 promoter were necessary for enhancing IL-8 promoter activity stimulated by TFF3. In fact, the expression of IL-8 is regulated by multiple transcription factors. Besides these transcription factors, TFF3 secreted from mammary carcinoma cells activated and increased tyrosine phosphorylation of STAT3. Depletion of STAT3 by siRNA partially abrogated the effect of TFF3 on IL-8 promoter activity and resulted in a small decrease of IL-8 expression. Hence, I postulate that STAT3 is one of a number of factors involved in the regulation of IL-8 expression stimulated by TFF3. Presumably, STAT3 is a transcription factor for IL-8 and cooperate with other transcription factors in the regulation of IL-8. TFF3 secreted from mammary carcinoma cells stimulated tyrosine phosphorylation of STAT3, the activated STAT3 dimerizes, and translocates into the nucleus to activate IL-8 gene expression (Bromberg *et al.*, 1999, Niu *et al.*, 2002, Rivat *et al.*,

2005). It has been reported that IL-6 induced STAT3 phosphorylation and increased the expression of TFF3 to promote cell migration and wound healing in biliary epithelial cells, which is through the activation of STAT3 and subsequent up-regulation of TFF3 expression (Jiang *et al.*, 2010). Additionally, TFF3 and VEGF-A function as promoters of colon cancer cell invasion, survival, and angiogenesis through STAT3-dependent signaling pathway (Rivat *et al.*, 2005). Earlier studies reported that VEGF-A activates STAT3, which in turn increased VEGF-A gene expression (Niu *et al.*, 1999, Niu *et al.*, 2002). It has been reported that TFF3 activated STAT3 activity to promote TFF3 gene transcription via autocrine loop (Rivat *et al.*, 2005). TFF3 and VEGFA have been demonstrated to activate STAT3 through tyrosine (Y705) phosphorylation of STAT3-α and STAT3-β isoforms (Bromberg *et al.*, 1999). TFF3 and VEGFA promoted cellular invasion and migration of colon cancer cells (Rivat *et al.*, 2005). Notably, TFF1 does not promote activation of STAT3, yet TFF1 was still capable to promote cellular invasion (Rivat *et al.*, 2005). Recently, TFF3 has been shown to promote invasion and metastasis of mammary carcinoma cells in Src-STAT3 dependent manner (Pandey *et al.*, 2014).

The constitutively active STAT3 increases VEGF-A expression and induces tumor angiogenesis in human pancreatic cancer cells. However, inhibition of STAT3 signaling with dominant-negative STAT3 inhibits VEGF-A expression, angiogenesis, and tumor growth (Wei *et al.*, 2003a). IL-6 stimulated VEGF-A protein expression in cervical cancer cells through activation of STAT3 phosphorylation. However, abrogation of the STAT3 pathway with a dominant-negative STAT3 protein reduced IL-6 stimulated tumor angiogenesis and the growth of cervical cancer cells (Wei *et al.*, 2003b). In accordance with these evidences, I have shown that STAT3 partially mediated the action of TFF3 on the stimulation of angiogenic behaviors of endothelial cells. Depletion of STAT3 by siRNA in mammary carcinoma cells with forced expression of TFF3 partially abrogated the effect of TFF3 on HUVEC migration, invasion and tubule formation *in vitro*. Mechanistically, TFF3 increased IL-8 expression and STAT3 phosphorylation that can partially elevated the expression of IL-8, which in turn

mediated the ability of TFF3 on the stimulation of angiogenic behaviors of endothelial cells via IL-8/CXCR2 axis. Therefore, TFF3 is a promoter of angiogenesis, which may also co-coordinate with the growth promoting and metastatic actions of TFF3 in mammary carcinoma to enhance tumor progression (Figure 59).

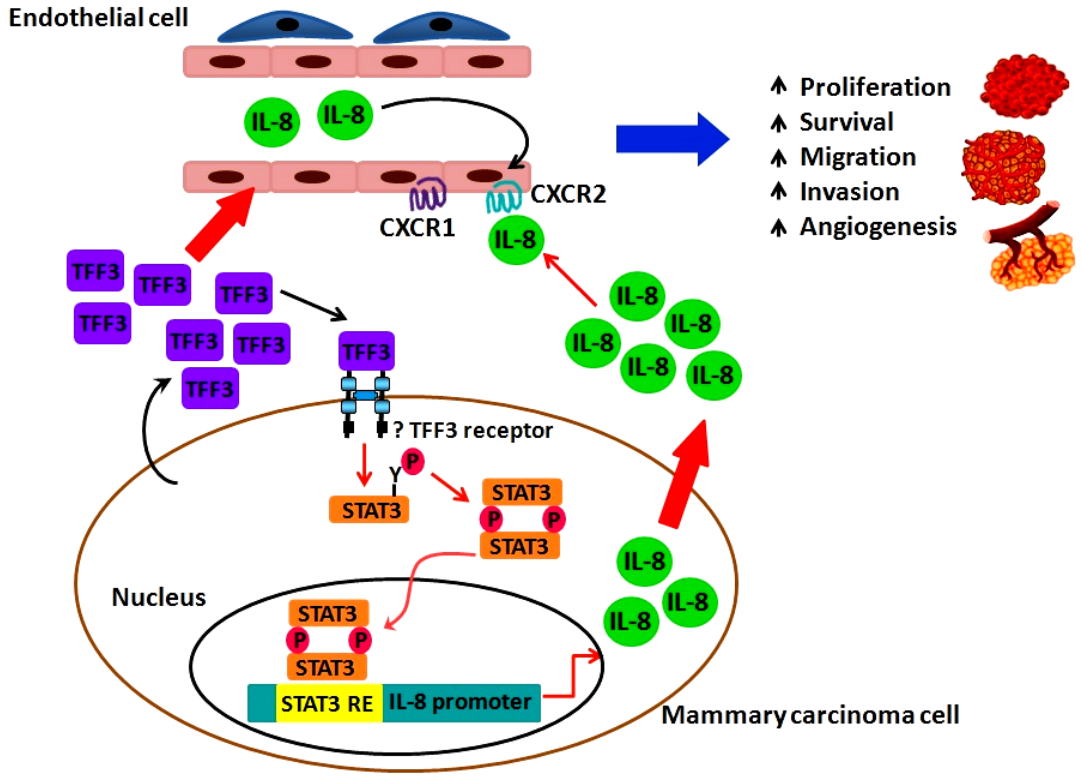


Figure 59: TFF3 is a promoter of angiogenesis in mammary carcinoma. TFF3 promoted IL-8 expression and increased tyrosine phosphorylation of STAT3 that is partially involved in the regulation of IL-8. The ability of TFF3 to stimulate angiogenic behaviors of HUVEC was mediated by IL-8 through CXCR2 axis. Furthermore, exogenous rhTFF3 directly acted on HUVEC to promote angiogenic behaviors of HUVEC. Additionally, TFF3 secreted from HUVEC promoted angiogenesis *in vitro* mediated by IL-8. Therefore, TFF3 is a promoter of angiogenesis, which may also co-coordinate with the growth promoting and metastatic actions of TFF3 in mammary carcinoma to enhance tumor progression.

In summary, I have demonstrated that depletion of TFF3 by siRNA in mammary carcinoma cells diminished the angiogenic behaviors of HUVEC. A previous study has reported that neutralization of TFF3 by anti-TFF3 polyclonal antibody reduced cell viability of mammary carcinoma cells and inhibited tumor growth in xenograft models (Kannan *et al.*, 2010). Collectively, inhibition of TFF3 by siRNA or anti-TFF3 antibody can be an effective

strategy to suppress tumor angiogenesis and metastasis in mammary carcinoma. Depletion of IL-8 by siRNA and inhibition IL-8 with anti-IL-8 monoclonal antibody abrogated the ability of TFF3 to stimulate angiogenic behaviors of HUVEC mediated by IL-8. Consistently, human anti-IL-8 antibody (e.g., ABX-IL8) has been reported to inhibit tumor angiogenesis, growth, and metastasis in bladder cancer and melanoma (Yang *et al.*, 1999, Huang *et al.*, 2002, Mian *et al.*, 2003). In addition, preclinical evaluation of the effectiveness of repertaxin (inhibitor of CXCR1/2) on animal models showed that mice treated with repertaxin developed fewer metastases than the mice treated with chemotherapy alone (Ginestier *et al.*, 2010). Therefore, the functional antagonism of TFF3 and/or IL-8 in mammary carcinoma cells could be a promising therapeutic strategy for suppression of the growth of TFF3-stumulated carcinomas.

5.5.2 Exogenous rhTFF3 stimulation and TFF3 secreted from HUVEC promoted angiogenesis

Herein, I have demonstrated that exogenous rhTFF3 stimulated HUVEC monolayer proliferation, migration, invasion, and tubule formation *in vitro* in concentration-dependent manner. Exogenous rhTFF3 directly acted on HUVEC to promote angiogenesis *in vitro.* Exogenous rhTFF3 may serve as both chemoattractant and stimulant to promote HUVEC migration and invasion. I suggested that TFF3 is a pro-angiogenic factor to promote angiogenic behaviors of endothelial cells through an autocrine loop. The angiogenic responsiveness of endothelial cells to the stimulation of TFF3 reflects the functional roles of TFF3 are unique, cell-type specific and implicated in different tissue of origin.

TFF3 secreted from HUVEC promoted monolayer cell proliferation, cell cycle progression, survival, migration, invasion, tubule formation *in vitro*. Depletion of TFF3 by siRNA in HUVEC decreased angiogenic behaviors of endothelial cells. TFF3 secreted from HUVEC exhibited similar functional effects as exogenous rhTFF3 stimulation. The protein expression of IL-8 in HUVEC was marginally increased by exogenous rhTFF3 stimulation. Additionally, TFF3 secreted from HUVEC was able to stimulate IL-8 promoter activity and

subsequently increased IL-8 expression, which in turn mediated the action of TFF3 to promote angiogenesis *in vitro* (Figure 59).

Considering therapeutic implications, both TFF3 siRNA and anti-sense TFF3 suppressed the growth and tumorigencity of mammary carcinoma (Kannan *et al.*, 2010). The anti-tumorigenic effect is mediated through induction of apoptosis and restored tamoxifen sensitivity in tamoxifen-resistance mammary carcinoma cells (Kannan *et al.*, 2010). TFF3 protein expression in tamoxifen-resistance mammary carcinoma cells was substantially increased and further induced by tamoxifen. Functional antagonism of TFF3 by antibody inhibited cell growth and improved tamoxifen sensitivity of tamoxifen-resistance mammary carcinoma cells (Kannan *et al.*, 2010). Over-expression of IL-8 is associated with a drug resistance phenotype (Duan *et al.*, 1999). Hence, neutralizing antibody IL-8 reversed the drug-resistance of MCF-7 cells to a taxol and doxorubicin. Inhibition of endogenous IL-8 by siRNA significantly enhanced the drug sensitivity of the drug resistant MCF-7 cells (Shi *et al.*, 2012). Additionally, depletion of IL-8 in prostate cancer increased the cytotoxic potency of a number of chemotherapeutic drugs (Singh and Lokeshwar, 2009). Therefore, anti-IL-8 antibody not only inhibit endothelial cell proliferation and capillary tube formation, but this neutralizing antibody also can restore the drug sensitivity of the doxorubicin resistant MCF-7 cells (Li *et al.*, 2005, Shi *et al.*, 2012). Hence, the functional antagonism of TFF3 and IL-8 would be an effective therapeutic approach to abolish the ability of TFF3 on the stimulation of *de novo* angiogenesis in mammary carcinoma.

5.2 Future direction

TFF3 is a small secreted protein and amenable to inhibition by antibody, inhibitor, and RNAi molecule. Several studies have attempted to delineate the potential of TFF antagonism as a cancer therapeutic. One study has recently employed anti-TFF3 polyclonal antibody to neutralize the secreted TFF3 from mammary carcinoma cells. Neutralization of TFF3 by anti-TFF3 polyclonal antibody reduced cell viability of mammary carcinoma cells and inhibited xenograft growth (Kannan *et al.*, 2010). The anti-TFF3 polyclonal antibody exhibited no potential adverse effects on the vital organs including liver, lung, intestine, kidney, and stomach in mice treated with anti-TFF3 polyclonal antibody when compared with mice treated with Ig-G control. Additionally, it has been reported that anti-sense TFF3 molecule inhibits the growth of gastric cancer cells, increases adriamycin-induced apoptosis, and enhances the response to chemotherapy (Chan *et al.*, 2005a). The anti-TFF3 polyclonal antibody and anti-sense TFF3 molecule can be utilized for studying the functional antagonism of TFF3 for suppression of *de novo* angiogenesis in mammary carcinoma. Alternatively, it is worth to develop inhibitor of TFF3 or neutralizing agent comprises RNAi molecule that can specifically target to TFF3 functional domain.

The effect of inhibition of TFF3 in mammary carcinoma cells by anti-TFF3 polyclonal antibody or inhibitor of TFF3 on the angiogenic activity of endothelial cells is required for further investigation. The *in vitro* functional antagonism study may define the effectiveness of anti-TFF3 polyclonal antibody to abrogate the effect of TFF3 in *de novo* angiogenesis in mammary carcinoma. The xenograft study may provide *in vivo* evidence to support that the functional antagonism of TFF3 by anti-TFF3 polyclonal antibody is capable to suppress tumor angiogenesis and metastasis of mammary carcinoma. This approach may be a novel therapeutic strategy for treatment of tumor angiogenesis stimulated by TFF3 in mammary carcinoma and it may also applicable for other neoplastic disorders. The efficacy, safety, and route of administration of TFF3 neutralizing agent or inhibitor of TFF3 are

required for further investigation and this agent may be of value in pharmacological intervention (Kjellev, 2009).

I have also demonstrated that TFF3 stimulated activation of STAT3 phosphorylation in mammary carcinoma cells. STAT3 partially increased the expression of IL-8 and partially mediated the effect of TFF3 on stimulation of angiogenic behaviors of endothelial cells. STAT3 is one of a number of transcription factors involved in the regulation of IL-8 expression. The mechanism by which TFF3 stimulated the activation of STAT3 in the regulation of IL-8 expression remains unclear. A study has reported that STAT3 may directly bind to the IL-8 promoter gene in the region from -272 to -133 bp (Oka *et al.*, 2010). A potential STAT3-binding sequence TTCACCAAA, consistent with the reported consensus sequences TT(N4/N5)AA, was identified from -245 to -237 bp in the 5′-flanking region of the IL-8 gene (Seidel *et al.*, 1995). Thereby, I propose that TFF3 stimulates activation of STAT3 and mediates direct binding of STAT3 to its consensus sites in IL-8 promoter. The potential STAT3-binding sites in IL-8 promoter would be validated by searching the 5'-flanking region of IL-8 gene for consensus STAT3-binding sites, TT(N_4)AA and TT(N_5)AA (Seidel *et al.*, 1995) using DNAStar sequence analysis software. The putative STAT3-binding site from -245 to -237 bp in the 5′-flanking region of human IL-8 gene cloned into the luciferase reporter plasmid pGL3-Basic and designated as wild-type construct. The mutation of individual base pairs in plasmid DNA (from 5'-TTCCCAAA-3' to 5'-GCGTCAAA-3') can be constructed according to site-specific mutagenesis procedure (Deng and Nickoloff, 1992) or using Site-Directed Mutagenesis Kit. The STAT3 mutant can be confirmed by sequencing. IL-8 promoter activity for STAT3 mutant will be assayed to determine if STAT3 is involved in the regulation of IL-8 expression stimulated by TFF3 and if STAT3 is one of the transcription factors required for activation of IL-8 expression. Furthermore, chromatin immunoprecipitation (ChIP) assays will be performed to determine if STAT3 directly bind to the STAT3-binding site in the IL-8 promoter *in vivo*. ChIP assay is commonly used for the identification of particular genomic DNA sequences that associated with a specific

transcription factor (Wells *et al.*, 2000). Thereby, the ChIP assay will validate if STAT3 directly bind to its consensus-binding site at the IL-8 promoter mediated by TFF3, IL-8 is the target gene of STAT3, and STAT3 is a transcription factor for IL-8. The other transcription factors for example, interferon regulatory factor-1 in the IL-8 promoter gene may be involved in the regulation of IL-8 mediated by TFF3 and these factors can be examined by the same approach.

The major challenge of future work is to identify the receptor(s) for TFF3 to mediate its functional effects in promoting of tumor growth and metastasis of cancers. Although a few of TFF3-binding proteins have recently been characterized, the receptor that mediates TFF3 signaling has not yet been identified (Otto and Thim, 2005). Many studies suggested that the existence of a TFF receptor, is localized at the basolateral cell membrane in epithelial cells, and is exposed only after injuries in the mucosal (Taupin and Podolsky, 2003, Hoffmann, 2009). However, the identity of this receptor is unknown. One study reported that the low-affinity chemokine receptor CXCR4 was identified to be a potential receptor for TFF2 (Dubeykovskaya *et al.*, 2009). As TFF3 are relatively similar to TFF2, it is rational that TFF3 should also be considered as potential ligand for that receptor as well (Hoffmann, 2009). The interaction between TFF3 and a TFF receptor on the membrane of epithelial cells is specific and as a typical binding of ligand-receptor (Yong *et al.*, 2013). The signaling pathways activated by the postulated receptors for TFF on the cell surface to the cytoplasm and/or nucleus remains to be determined. Several studies have proposed that the possible contribution of APC/E-cadherin/β-catenins, EGF receptors and MAPK as downstream signaling pathways activated by TFF peptides, leading to decreased cell-cell contact and cell-substratum adhesion (Liu *et al.*, 1997, Efstathiou *et al.*, 1998). It has been reported that signaling pathways including Src/RhoA, PI3K/Akt, and phospholipase C/PKC are mediated by TFF peptides to promote cellular invasion and survival of colon carcinoma cells (Emami *et al.*, 2001). Many bioinformatics software are available and can be used to integrate microarray data and *in vitro* experimental data of the functionality of a targeted gene. For

example, DAVID bioinformatics resources is a feasible screening tool for highlighting the functional domain and motif of TFF3 protein, predicting interacting protein to the trefoil domain of TFF3, and integrating the functionality of TFF3 with the downstream signaling pathways. KEGG pathway maps can display the interconnection between TFF3 and the signaling proteins implicated in promoting or inhibiting of tumor growth and progression. The information generated from these tools is required for further validation by *in vitro* and *in vivo* experimental studies. Collaboration researchers from different discipline (bioinformatics and cancer biology) will build up a multi-discipline platform to identify TFF3 interacting proteins on the surface of mammary carcinoma cell lines (Lei *et al.*, 2012). Identification of TFF3 receptor(s) can provide a better understanding of TFF3 biology and develop a novel drug targets for mammary carcinoma progression.

Bibliography

1. Achen MG, Mann GB, Stacker SA. Targeting lymphangiogenesis to prevent tumour metastasis. *Br J Cancer* 2006, 94(10):1355-1360.

2. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 2009, 119(6):1438-1449.

3. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 2007, 8(6):464-478.

4. Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* 2000, 165(9):5269-5277.

5. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *The Journal of investigative dermatology* 1992, 99(6):683-690.

6. Ahmed ARH, Griffiths AB, Tilby MT, Westley BR, May FEB. TFF3 Is a Normal Breast Epithelial Protein and Is Associated with Differentiated Phenotype in Early Breast Cancer but Predisposes to Invasion and Metastasis in Advanced Disease. *The American journal of pathology* 2012, 180(3):904-916.

7. Ahuja SK, Murphy PM. The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. *J Biol Chem* 1996, 271(34):20545-20550.

8. Al-azzeh E, Dittrich O, Vervoorts J, Blin N, Gott P, Luscher B. Gastroprotective peptide trefoil factor family 2 gene is activated by upstream stimulating factor but not by c-Myc in gastrointestinal cancer cells. *Gut* 2002, 51(5):685-690.

9. Al-azzeh ED, Fegert P, Blin N, Gott P. Transcription factor GATA-6 activates expression of gastroprotective trefoil genes TFF1 and TFF2. *Biochim Biophys Acta* 2000, 1490(3):324-332.

10. Al-Kuraya K, Schraml P, Torhorst J, Tapia C, Zaharieva B, Novotny H, Spichtin H, Maurer R, Mirlacher M, Kochli O, Zuber M, Dieterich H, Mross F, Wilber K, Simon R, Sauter G. Prognostic relevance of gene amplifications and coamplifications in breast cancer. *Cancer research* 2004, 64(23):8534-8540.

11. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer* 2002, 2(2):101-112.

12. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature* 2005, 438(7070):946-953.

13. Allred DC, Mohsin SK, Fuqua SA. Histological and biological evolution of human premalignant breast disease. *Endocr Relat Cancer* 2001, 8(1):47-61.

14. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *Journal of molecular biology* 1990, 215(3):403-410.

15. American Cancer Society. Cancer Facts & Figures 2014. Atlanta: American Cancer Society. 2014.

16. Amiry N, Kong X, Muniraj N, Kannan N, Grandison PM, Lin J, Yang Y, Vouyovitch CM, Borges S, Perry JK, Mertani HC, Zhu T, Liu D, Lobie PE. Trefoil factor-1 (TFF1) enhances oncogenicity of mammary carcinoma cells. *Endocrinology* 2009, 150(10):4473-4483.

17. Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB. Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical research* 2008, 25(9):2097-2116.

18. Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. *Breast Cancer Res* 2002, 4(5):197-201.

19. Andre T, Kotelevets L, Vaillant JC, Coudray AM, Weber L, Prevot S, Parc R, Gespach C, Chastre E. Vegf, Vegf-B, Vegf-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *International journal of cancer Journal international du cancer* 2000, 86(2):174-181.

20. Aranda E, Owen GI. A semi-quantitative assay to screen for angiogenic compounds and compounds with angiogenic potential using the EA.hy926 endothelial cell line. *Biological research* 2009, 42(3):377-389.

21. Araneda S, Pelloux S, Radicella JP, Angulo J, Kitahama K, Gysling K, Forray MI. 8-oxoguanine DNA glycosylase, but not Kin17 protein, is translocated and differentially regulated by estrogens in rat brain cells. *Neuroscience* 2005, 136(1):135-146.

22. Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, Frank D, Brownlee M, Flynn E, Parangi S, Byers HR, Folkman J. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. *Proceedings of the National Academy of Sciences* 1997, 94(3):861-866.

23. Asselin-Labat M-L, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ. Steroid Hormone Receptor Status of Mouse Mammary Stem Cells. *Journal of the National Cancer Institute* 2006, 98(14):1011-1014.

24. Atwood CS, Hovey RC, Glover JP, Chepko G, Ginsburg E, Robison WG, Vonderhaar BK. Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. *The Journal of endocrinology* 2000, 167(1):39-52.

25. Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis Assays: A Critical Overview. *Clinical Chemistry* 2003, 49(1):32-40.

26. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol* 2009, 10(3):165-177.

27. Babyatsky M, Lin J, Yio X, Chen A, Zhang JY, Zheng Y, Twyman C, Bao X, Schwartz M, Thung S, Lawrence Werther J, Itzkowitz S. Trefoil factor-3 expression in human colon cancer liver metastasis. *Clin Exp Metastasis* 2009, 26(2):143-151.

28. Bacac M, Stamenkovic I. Metastatic Cancer Cell. *Annual Review of Pathology: Mechanisms of Disease* 2008, 3(1):221-247.

29. Baeriswyl V, Christofori G. The angiogenic switch in carcinogenesis. *Semin Cancer Biol* 2009, 19(5):329-337.

30. Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Annual review of immunology* 1997, 15:675-705.

31. Bagley RG, Walter-Yohrling J, Cao X, Weber W, Simons B, Cook BP, Chartrand SD, Wang C, Madden SL, Teicher BA. Endothelial precursor cells as a model of tumor endothelium: characterization and comparison with mature endothelial cells. *Cancer research* 2003, 63(18):5866-5873.

32. Baish JW, Jain RK. Fractals and Cancer. *Cancer research* 2000, 60(14):3683-3688.

33. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Current opinion in genetics & development* 2005, 15(1):102-111.

34. Banerjee A, Wu ZS, Qian PX, Kang J, Liu DX, Zhu T, Lobie PE. ARTEMIN promotes de novo angiogenesis in ER negative mammary carcinoma through activation of TWIST1-VEGF-A signalling. *PLoS One* 2012, 7(11):e50098.

35. Banerjee A, Wu ZS, Qian P, Kang J, Pandey V, Liu DX, Zhu T, Lobie PE. ARTEMIN synergizes with TWIST1 to promote metastasis and poor survival outcome in patients with ER negative mammary carcinoma. *Breast Cancer Res* 2011, 13(6):R112.

36. Banerjee S, Dowsett M, Ashworth A, Martin LA. Mechanisms of disease: angiogenesis and the management of breast cancer. *Nat Clin Pract Oncol* 2007, 4(9):536-550.

37. Barkhem T, Haldosen LA, Gustafsson JA, Nilsson S. pS2 Gene expression in HepG2 cells: complex regulation through crosstalk between the estrogen receptor alpha, an estrogen-responsive element, and the activator protein 1 response element. *Molecular pharmacology* 2002a, 61(6):1273-1283.

38. Barkhem T, Haldosen LA, Gustafsson JA, Nilsson S. Transcriptional synergism on the pS2 gene promoter between a p160 coactivator and estrogen receptor-alpha depends on the coactivator subtype, the type of estrogen response element, and the promoter context. *Molecular endocrinology (Baltimore, Md)* 2002b, 16(11):2571-2581.

39. Baselga J, Norton L. Focus on breast cancer. *Cancer Cell* 2002, 1(4):319-322.

40. Basolo F, Conaldi PG, Fiore L, Calvo S, Toniolo A. Normal breast epithelial cells produce interleukins 6 and 8 together with tumor-necrosis factor: defective IL6 expression in mammary carcinoma. *International journal of cancer Journal international du cancer* 1993, 55(6):926-930.

41. Battegay EJ, Rupp J, Iruela-Arispe L, Sage EH, Pech M. PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors. *J Cell Biol* 1994, 125(4):917-928.

42. Baum B, Settleman J, Quinlan MP. Transitions between epithelial and mesenchymal states in development and disease. *Seminars in cell & developmental biology* 2008, 19(3):294-308.

43. Baus-Loncar M, Al-azzeh ED, Romanska H, Lalani el N, Stamp GW, Blin N, Kayademir T. Transcriptional control of TFF3 (intestinal trefoil factor) via promoter binding sites for the nuclear factor kappaB and C/EBPbeta. *Peptides* 2004, 25(5):849-854.

44. Beck S, Sommer P, dos Santos Silva E, Blin N, Gott P. Hepatocyte nuclear factor 3 (winged helix domain) activates trefoil factor gene TFF1 through a binding motif adjacent to the TATAA box. *DNA and cell biology* 1999, 18(2):157-164.

45. Beck S, Schmitt H, Shizuya H, Blin N, Gott P. Cloning of contiguous genomic fragments from human chromosome 21 harbouring three trefoil peptide genes. *Human genetics* 1996, 98(2):233-235.

46. Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA, Bender HG. Multistep carcinogenesis of breast cancer and tumour heterogeneity. *Journal of molecular medicine (Berlin, Germany)* 1997, 75(6):429-439.

47. Beierle EA, Strande LF, Berger AC, Chen MK. VEGF is upregulated in a neuroblastoma and hepatocyte coculture model. *The Journal of surgical research* 2001, 97(1):34-40.

48. Bengtsson AK, Ryan EJ, Giordano D, Magaletti DM, Clark EA. 17beta-estradiol (E2) modulates cytokine and chemokine expression in human monocyte-derived dendritic cells. *Blood* 2004, 104(5):1404-1410.

49. Benoy IH, Salgado R, Van Dam P, Geboers K, Van Marck E, Scharpe S, Vermeulen PB, Dirix LY. Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. *Clin Cancer Res* 2004, 10(21):7157-7162.

50. Beral V, Collaborative Group on Hormonal Factors in Breast C. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. *Lancet* 1997, 350(9084):1047-1059.

51. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003, 3(6):401-410.

52. Berry M, Nunez AM, Chambon P. Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. *Proc Natl Acad Sci U S A* 1989, 86(4):1218-1222.

53. Bignotti E, Ravaggi A, Tassi RA, Calza S, Rossi E, Falchetti M, Romani C, Bandiera E, Odicino FE, Pecorelli S, Santin AD. Trefoil factor 3: a novel serum marker identified by gene expression profiling in high-grade endometrial carcinomas. *Br J Cancer* 2008, 99(5):768-773.

54. Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NF, Wheatley DN. An in vitro model of angiogenesis: basic features. *Angiogenesis* 1999, 3(4):335-344.

55. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med* 2011, 17(3):320-329.

56. Bjorndahl MA, Cao R, Burton JB, Brakenhielm E, Religa P, Galter D, Wu L, Cao Y. Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. *Cancer research* 2005, 65(20):9261-9268.

57. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkila P, Heikkinen T, Nevanlinna H, Akslen LA, Begin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE, Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E, Dawson SJ, Dunning AM, Humphreys M, Easton DF, Garcia-Closas M, Caldas C, Pharoah PD, Huntsman D. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. *PLoS medicine* 2010, 7(5):e1000279.

58. Bocchinfuso WP, Lindzey JK, Hewitt SC, Clark JA, Myers PH, Cooper R, Korach KS. Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology* 2000, 141(8):2982-2994.

59. Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 1998, 19(3):225-268.

60. Bonkhoff H, Stein U, Welter C, Remberger K. Differential expression of the pS2 protein in the human prostate and prostate cancer: association with premalignant changes and neuroendocrine differentiation. *Hum Pathol* 1995, 26(8):824-828.

61. Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, Smith SK. Determination of the transcript profile of human endometrium. *Molecular human reproduction* 2003, 9(1):19-33.

62. Bosma AJ, Weigelt B, Lambrechts AC, Verhagen OJ, Pruntel R, Hart AA, Rodenhuis S, van 't Veer LJ. Detection of circulating breast tumor cells by differential expression of marker genes. *Clin Cancer Res* 2002, 8(6):1871-1877.

63. Bossenmeyer-Pourie C, Kannan R, Ribieras S, Wendling C, Stoll I, Thim L, Tomasetto C, Rio MC. The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. *J Cell Biol* 2002, 157(5):761-770.

64. Bouck N, Stellmach V, Hsu SC. How tumors become angiogenic. *Adv Cancer Res* 1996, 69:135-174.

65. Bouis D, Hospers GA, Meijer C, Molema G, Mulder NH. Endothelium in vitro: a review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis* 2001, 4(2):91-102.

66. Bouïs D, Hospers G, Meijer C, Molema G, Mulder N. Endothelium in vitro: A review of human vascular endothelial cell lines for blood vessel-related research. *Angiogenesis* 2001, 4(2):91-102.

67. Brasier AR, Jamaluddin M, Casola A, Duan W, Shen Q, Garofalo RP. A promoter recruitment mechanism for tumor necrosis factor-alpha-induced interleukin-8 transcription in type II pulmonary epithelial cells. Dependence on nuclear abundance of Rel A, NF-kappaB1, and c-Rel transcription factors. *The Journal of biological chemistry* 1998, 273(6):3551-3561.

68. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-oncology* 2005, 7(2):122-133.

69. Brenton JD, Carey LA, Ahmed AA, Caldas C. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? *J Clin Oncol* 2005, 23(29):7350-7360.

70. Brew R, Erikson JS, West DC, Kinsella AR, Slavin J, Christmas SE. Interleukin-8 as an autocrine growth factor for human colon carcinoma cells in vitro. *Cytokine* 2000, 12(1):78-85.

71. Brisken C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA. A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proceedings of the National Academy of Sciences* 1998, 95(9):5076-5081.

72. Bromberg J, Darnell JE, Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000, 19(21):2468-2473.

73. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr. Stat3 as an oncogene. *Cell* 1999, 98(3):295-303.

74. Brosh R, Rotter V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* 2009, 9(10):701-713.

75. Burns KA, Korach KS. Estrogen receptors and human disease: an update. *Archives of toxicology* 2012, 86(10):1491-1504.

76. Calnan DP, Westley BR, May FE, Floyd DN, Marchbank T, Playford RJ. The trefoil peptide TFF1 inhibits the growth of the human gastric adenocarcinoma cell line AGS. *The Journal of pathology* 1999, 188(3):312-317.

77. Cameron IL, Short N, Sun L, Hardman WE. Endothelial cell pseudopods and angiogenesis of breast cancer tumors. *Cancer Cell Int* 2005, 5(1):17.

78. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012, 490(7418):61-70.

79. Cao Y. Emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. *Nat Rev Cancer* 2005, 5(9):735-743.

80. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC. Race, breast cancer subtypes, and survival in

the Carolina Breast Cancer Study. *JAMA : the journal of the American Medical Association* 2006, 295(21):2492-2502.

81. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000, 407(6801):249-257.

82. Carrasco R, Pera M, May FE, Westley BR, Martinez A, Morales L. Trefoil factor family peptide 3 prevents the development and promotes healing of ischemia-reperfusion injury in weanling rats. *Journal of pediatric surgery* 2004, 39(11):1693-1700.

83. Carvalho R, Kayademir T, Soares P, Canedo P, Sousa S, Oliveira C, Leistenschneider P, Seruca R, Gott P, Blin N, Carneiro F, Machado JC. Loss of heterozygosity and promoter methylation, but not mutation, may underlie loss of TFF1 in gastric carcinoma. *Lab Invest* 2002, 82(10):1319-1326.

84. Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 2005, 8(4):299-309.

85. Cavallaro U, Schaffhauser B, Christofori G. Cadherins and the tumour progression: is it all in a switch? *Cancer Lett* 2002, 176(2):123-128.

86. Cerretti DP, Kozlosky CJ, Vanden Bos T, Nelson N, Gearing DP, Beckmann MP. Molecular characterization of receptors for human interleukin-8, GRO/melanoma growth-stimulatory activity and neutrophil activating peptide-2. *Molecular immunology* 1993, 30(4):359-367.

87. Chan MW, Chan VY, Leung WK, Chan KK, To KF, Sung JJ, Chan FK. Anti-sense trefoil factor family-3 (intestinal trefoil factor) inhibits cell growth and induces chemosensitivity to adriamycin in human gastric cancer cells. *Life Sci* 2005a, 76(22):2581-2592.

88. Chan VY, Chan MW, Leung WK, Leung PS, Sung JJ, Chan FK. Intestinal trefoil factor promotes invasion in non-tumorigenic Rat-2 fibroblast cell. *Regul Pept* 2005b, 127(1-3):87-94.

89. Charames GS, Bapat B. Genomic instability and cancer. *Current molecular medicine* 2003, 3(7):589-596.

90. Chen Y, Olopade OI. MYC in breast tumor progression. *Expert review of anticancer therapy* 2008, 8(10):1689-1698.

91. Chen YH, Lu Y, De Plaen IG, Wang LY, Tan XD. Transcription factor NF-kappaB signals antianoikic function of trefoil factor 3 on intestinal epithelial cells. *Biochem Biophys Res Commun* 2000, 274(3):576-582.

92. Cheng GZ, Park S, Shu S, He L, Kong W, Zhang W, Yuan Z, Wang LH, Cheng JQ. Advances of AKT pathway in human oncogenesis and as a target for anti-cancer drug discovery. *Current cancer drug targets* 2008, 8(1):2-6.

93. Chinery R, Playford RJ. Combined intestinal trefoil factor and epidermal growth factor is prophylactic against indomethacin-induced gastric damage in the rat. *Clinical science (London, England : 1979)* 1995, 88(4):401-403.

94. Chinery R, Cox HM. Immunoprecipitation and characterization of a binding protein specific for the peptide, intestinal trefoil factor. *Peptides* 1995, 16(4):749-755.

95. Christofori G, Semb H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci* 1999, 24(2):73-76.

96. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G, Slamon DJ. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999, 17(9):2639-2648.

97. Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. *Cardiovascular research* 2001, 49(3):507-521.

98. Cook GA, Familari M, Thim L, Giraud AS. The trefoil peptides TFF2 and TFF3 are expressed in rat lymphoid tissues and participate in the immune response. *FEBS Lett* 1999, 456(1):155-159.

99. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science (New York, NY)* 1985, 230(4730):1132-1139.

100. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. *Chem Biol* 1996, 3(11):895-904.

101. Cowley SM, Hoare S, Mosselman S, Parker MG. Estrogen receptors alpha and beta form heterodimers on DNA. *J Biol Chem* 1997, 272(32):19858-19862.

102. Crivellato E. The role of angiogenic growth factors in organogenesis. *The International journal of developmental biology* 2011, 55(4-5):365-375.

103. Crown J, O'Shaughnessy J, Gullo G. Emerging targeted therapies in triple-negative breast cancer. *Annals of Oncology* 2012, 23(suppl 6):vi56-vi65.

104. Czabotar PE, Colman PM, Huang DC. Bax activation by Bim? *Cell death and differentiation* 2009, 16(9):1187-1191.

105. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, Maggi A, Muramatsu M, Parker MG, Gustafsson JA. International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacological reviews* 2006, 58(4):773-781.

106. Dalal BI, Keown PA, Greenberg AH. Immunocytochemical localization of secreted transforming growth factor-beta 1 to the advancing edges of primary tumors and to lymph node metastases of human mammary carcinoma. *The American journal of pathology* 1993, 143(2):381-389.

107. Daniel TO, Abrahamson D. Endothelial signal integration in vascular assembly. *Annual review of physiology* 2000, 62:649-671.

108. Darnell JE, Jr. STATs and gene regulation. *Science (New York, NY)* 1997, 277(5332):1630-1635.

109. Dawood S, Hu R, Homes MD, Collins LC, Schnitt SJ, Connolly J, Colditz GA, Tamimi RM. Defining breast cancer prognosis based on molecular phenotypes: results from a large cohort study. *Breast Cancer Res Treat* 2011, 126(1):185-192.

110. De Larco JE, Wuertz BR, Yee D, Rickert BL, Furcht LT. Atypical methylation of the interleukin-8 gene correlates strongly with the metastatic potential of breast carcinoma cells. *Proc Natl Acad Sci U S A* 2003, 100(24):13988-13993.

111. De Larco JE, Wuertz BR, Rosner KA, Erickson SA, Gamache DE, Manivel JC, Furcht LT. A potential role for interleukin-8 in the metastatic phenotype of breast carcinoma cells. *The American journal of pathology* 2001, 158(2):639-646.

112. Deming SL, Nass SJ, Dickson RB, Trock BJ. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br J Cancer* 2000, 83(12):1688-1695.

113. Deng WP, Nickoloff JA. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Analytical biochemistry* 1992, 200(1):81-88.

114. Devalaraja RM, Nanney LB, Du J, Qian Q, Yu Y, Devalaraja MN, Richmond A. Delayed wound healing in CXCR2 knockout mice. *The Journal of investigative dermatology* 2000, 115(2):234-244.

115. Dhar DK, Wang TC, Tabara H, Tonomoto Y, Maruyama R, Tachibana M, Kubota H, Nagasue N. Expression of trefoil factor family members correlates with patient prognosis and neoangiogenesis. *Clin Cancer Res* 2005, 11(18):6472-6478.

116. Dickson RB, Bates SE, McManaway ME, Lippman ME. Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer research* 1986, 46(4 Pt 1):1707-1713.

117. Dignass A, Lynch-Devaney K, Kindon H, Thim L, Podolsky DK. Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *J Clin Invest* 1994, 94(1):376-383.

118. Doane AS, Danso M, Lal P, Donaton M, Zhang L, Hudis C, Gerald WL. An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. *Oncogene* 2006, 25(28):3994-4008.

119. Donovan D, Brown NJ, Bishop ET, Lewis CE. Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo. *Angiogenesis* 2001, 4(2):113-121.

120. Dossinger V, Kayademir T, Blin N, Gott P. Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2002, 12(4):197-206.

121. Duan Z, Feller AJ, Penson RT, Chabner BA, Seiden MV. Discovery of differentially expressed genes associated with paclitaxel resistance using cDNA array technology: analysis of interleukin (IL) 6, IL-8, and monocyte chemotactic protein 1 in the paclitaxel-resistant phenotype. *Clinical cancer research : an official journal of the American Association for Cancer Research* 1999, 5(11):3445-3453.

122. Dubeykovskaya Z, Dubeykovskiy A, Solal-Cohen J, Wang TC. Secreted trefoil factor 2 activates the CXCR4 receptor in epithelial and lymphocytic cancer cell lines. *J Biol Chem* 2009, 284(6):3650-3662.

123. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002, 20(21):4368-4380.

124. Dvorak HF, Weaver VM, Tlsty TD, Bergers G. Tumor microenvironment and progression. *Journal of Surgical Oncology* 2011, 103(6):468-474.

125. Efstathiou JA, Liu D, Wheeler JM, Kim HC, Beck NE, Ilyas M, Karayiannakis AJ, Mortensen NJ, Kmiot W, Playford RJ, Pignatelli M, Bodmer WF. Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells. *Proc Natl Acad Sci U S A* 1999, 96(5):2316-2321.

126. Efstathiou JA, Noda M, Rowan A, Dixon C, Chinery R, Jawhari A, Hattori T, Wright NA, Bodmer WF, Pignatelli M. Intestinal trefoil factor controls the expression of the adenomatous polyposis coli-catenin and the E-cadherin-catenin complexes in human colon carcinoma cells. *Proc Natl Acad Sci U S A* 1998, 95(6):3122-3127.

127. Egeblad M, Littlepage LE, Werb Z. The fibroblastic coconspirator in cancer progression. *Cold Spring Harbor symposia on quantitative biology* 2005, 70:383-388.

128. Ehmann UK, Stevenson MA, Calderwood SK, DeVries JT. Physical connections between feeder cells and recipient normal mammary epithelial cells. *Experimental cell research* 1998, 243(1):76-86.

129. Ehrmann RL, Knoth M. Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy. *J Natl Cancer Inst* 1968, 41(6):1329-1341.

130. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* 2008, 8(8):579-591.

131. Ellsworth RE, Hooke JA, Love B, Kane JL, Patney HL, Ellsworth DL, Shriver CD. Correlation of levels and patterns of genomic instability with histological grading of invasive breast tumors. *Breast Cancer Res Treat* 2008a, 107(2):259-265.

132. Ellsworth RE, Ellsworth DL, Patney HL, Deyarmin B, Love B, Hooke JA, Shriver CD. Amplification of HER2 is a marker for global genomic instability. *BMC cancer* 2008b, 8:297.

133. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991, 19(5):403-410.

134. Emami S, Rodrigues S, Rodrigue CM, Le Floch N, Rivat C, Attoub S, Bruyneel E, Gespach C. Trefoil factor family (TFF) peptides and cancer progression. *Peptides* 2004, 25(5):885-898.

135. Emami S, Le Floch N, Bruyneel E, Thim L, May F, Westley B, Rio M, Mareel M, Gespach C. Induction of scattering and cellular invasion by trefoil peptides in src-

and RhoA-transformed kidney and colonic epithelial cells. *FASEB J* 2001, 15(2):351-361.

136. Erler JT, Weaver VM. Three-dimensional context regulation of metastasis. *Clin Exp Metastasis* 2009, 26(1):35-49.

137. Ewington L, Taylor A, Sriraksa R, Horimoto Y, Lam EW, El-Bahrawy MA. The expression of interleukin-8 and interleukin-8 receptors in endometrial carcinoma. *Cytokine* 2012, 59(2):417-422.

138. Fabian CJ, Kimler BF. Selective estrogen-receptor modulators for primary prevention of breast cancer. *J Clin Oncol* 2005, 23(8):1644-1655.

139. Faith DA, Isaacs WB, Morgan JD, Fedor HL, Hicks JL, Mangold LA, Walsh PC, Partin AW, Platz EA, Luo J, De Marzo AM. Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays. *Prostate* 2004, 61(3):215-227.

140. Faivre S, Regnauld K, Bruyneel E, Nguyen QD, Mareel M, Emami S, Gespach C. Suppression of cellular invasion by activated G-protein subunits Galphao, Galphai1, Galphai2, and Galphai3 and sequestration of Gbetagamma. *Molecular pharmacology* 2001, 60(2):363-372.

141. Fan F, Wood KV. Bioluminescent assays for high-throughput screening. *Assay and drug development technologies* 2007, 5(1):127-136.

142. Fedi P, Tronick SR, Aaronson SA. Growth factors In: *Cancer Medicine.* Holland JF, Bast RC, Morton DL, Frei E, Kufe DW, Weichselbaum RR, eds. B.C. Decker Inc.: Hamilton, 2000: pp 41-64.

143. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin D, Forman D, Bray F. GLOBOCAN 2012 v1.0 , Cancer Incidence and Mortality Worldwide: IARC Cancer. 2012.

144. Fernando H, Chin C, Rösgen J, Rajarathnam K. Dimer Dissociation Is Essential for Interleukin-8 (IL-8) Binding to CXCR1 Receptor. *Journal of Biological Chemistry* 2004, 279(35):36175-36178.

145. Ferrara N. The role of VEGF in the regulation of physiological and pathological angiogenesis. *Exs* 2005(94):209-231.

146. Ferrara N. Vascular Endothelial Growth Factor as a Target for Anticancer Therapy. *The Oncologist* 2004, 9(suppl 1):2-10.

147. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003, 9(6):669-676.

148. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003, 3(6):453-458.

149. Finger EC, Giaccia AJ. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer metastasis reviews* 2010, 29(2):285-293.

150. Floor SL, Dumont JE, Maenhaut C, Raspe E. Hallmarks of cancer: of all cancer cells, all the time? *Trends in molecular medicine* 2012, 18(9):509-515.

151. Folkman J. Tumor angiogenesis. *Adv Cancer Res* 1985, 43:175-203.

152. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* 1971, 133(2):275-288.

153. Folkman J. Fundamental concepts of the angiogenic process. *Current molecular medicine* 2003, 3(7):643-651.

154. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990, 82(1):4-6.

155. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992, 3(2):65-71.

156. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971, 285(21):1182-1186.

157. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995, 1(1):27-31.

158. Forster C, Makela S, Warri A, Kietz S, Becker D, Hultenby K, Warner M, Gustafsson JA. Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. *Proc Natl Acad Sci U S A* 2002, 99(24):15578-15583.

159. Fox SB, Generali DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res* 2007, 9(6):216.

160. Freund A, Chauveau C, Brouillet JP, Lucas A, Lacroix M, Licznar A, Vignon F, Lazennec G. IL-8 expression and its possible relationship with estrogen-receptor-negative status of breast cancer cells. *Oncogene* 2003, 22(2):256-265.

161. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003, 3(5):362-374.

162. Friesel RE, Maciag T. Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. *Faseb j* 1995, 9(10):919-925.

163. Fujimoto J, Yasui W, Tahara H, Tahara E, Kudo Y, Yokozaki H, Tahara E. DNA hypermethylation at the pS2 promoter region is associated with early stage of stomach carcinogenesis. *Cancer Lett* 2000, 149(1-2):125-134.

164. Fujisawa N, Sakao Y, Hayashi S, Hadden WA, 3rd, Harmon CL, Miller EJ. alpha-Chemokine growth factors for adenocarcinomas; a synthetic peptide inhibitor for alpha-chemokines inhibits the growth of adenocarcinoma cell lines. *Journal of cancer research and clinical oncology* 2000, 126(1):19-26.

165. Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford K, Narravula S, Podolsky DK, Colgan SP. Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. *J Exp Med* 2001, 193(9):1027-1034.

166. Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R, Rahman N, Stratton MR. A census of human cancer genes. *Nat Rev Cancer* 2004, 4(3):177-183.

167. Gadkar-Sable S, Shah C, Rosario G, Sachdeva G, Puri C. Progesterone receptors: various forms and functions in reproductive tissues. *Frontiers in bioscience : a journal and virtual library* 2005, 10:2118-2130.

168. Garcia R, Bowman TL, Niu G, Yu H, Minton S, Muro-Cacho CA, Cox CE, Falcone R, Fairclough R, Parsons S, Laudano A, Gazit A, Levitzki A, Kraker A, Jove R. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene* 2001, 20(20):2499-2513.

169. Gasparini G, Longo R, Toi M, Ferrara N. Angiogenic inhibitors: a new therapeutic strategy in oncology. *Nat Clin Pract Oncol* 2005, 2(11):562-577.

170. Gelao L, Criscitiello C, Fumagalli L, Locatelli M, Manunta S, Esposito A, Minchella I, Goldhirsch A, Curigliano G. Tumour dormancy and clinical implications in breast cancer. *Ecancermedicalscience* 2013, 7:320.

171. Gharavi NM, Alva JA, Mouillesseaux KP, Lai C, Yeh M, Yeung W, Johnson J, Szeto WL, Hong L, Fishbein M, Wei L, Pfeffer LM, Berliner JA. Role of the Jak/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids in vitro and in atherosclerosis in vivo. *J Biol Chem* 2007, 282(43):31460-31468.

172. Ginestier C, Liu S, Diebel ME, Korkaya H, Luo M, Brown M, Wicinski J, Cabaud O, Charafe-Jauffret E, Birnbaum D, Guan JL, Dontu G, Wicha MS. CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J Clin Invest* 2010, 120(2):485-497.

173. Goiot H, Attoub S, Kermorgant S, Laigneau JP, Lardeux B, Lehy T, Lewin MJ, Bado A. Antral mucosa expresses functional leptin receptors coupled to STAT-3 signaling, which is involved in the control of gastric secretions in the rat. *Gastroenterology* 2001, 121(6):1417-1427.

174. Göke MN, Cook JR, Kunert KS, Fini ME, Gipson IK, Podolsky DK. Trefoil peptides promote restitution of wounded corneal epithelial cells. *Experimental cell research* 2001, 264(2):337-344.

175. Gomperts BN, Strieter RM. Chemokine-directed metastasis. *Contributions to microbiology* 2006, 13:170-190.

176. Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A* 1990, 87(17):6624-6628.

177. Graham JD, Yager ML, Hill HD, Byth K, O'Neill GM, Clarke CL. Altered progesterone receptor isoform expression remodels progestin responsiveness of breast cancer cells. *Molecular endocrinology (Baltimore, Md)* 2005, 19(11):2713-2735.

178. Graness A, Chwieralski CE, Reinhold D, Thim L, Hoffmann W. Protein kinase C and ERK activation are required for TFF-peptide-stimulated bronchial epithelial cell

migration and tumor necrosis factor-alpha-induced interleukin-6 (IL-6) and IL-8 secretion. *J Biol Chem* 2002, 277(21):18440-18446.

179. Green AR, Green VL, White MC, Speirs V. Expression of cytokine messenger RNA in normal and neoplastic human breast tissue: identification of interleukin-8 as a potential regulatory factor in breast tumours. *International journal of cancer Journal international du cancer* 1997, 72(6):937-941.

180. Green J, Cairns BJ, Casabonne D, Wright FL, Reeves G, Beral V. Height and cancer incidence in the Million Women Study: prospective cohort, and meta-analysis of prospective studies of height and total cancer risk. *The lancet oncology* 2011, 12(8):785-794.

181. Greenblatt M, Shubi P. Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique. *J Natl Cancer Inst* 1968, 41(1):111-124.

182. Gruvberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meltzer PS. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. *Cancer research* 2001, 61(16):5979-5984.

183. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene* 2006, 25(43):5864-5874.

184. Gupta MK, Qin RY. Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* 2003, 9(6):1144-1155.

185. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000, 100(1):57-70.

186. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, 86(3):353-364.

187. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011, 144(5):646-674.

188. Hanby AM, Poulsom R, Singh S, Elia G, Jeffery RE, Wright NA. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. *Gastroenterology* 1993, 105(4):1110-1116.

189. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *Journal of leukocyte biology* 1994, 56(5):559-564.

190. Harris CC. p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis* 1996, 17(6):1187-1198.

191. Harvey K, Welch Z, Kovala AT, Garcia JG, English D. Comparative analysis of in vitro angiogenic activities of endothelial cells of heterogeneous origin. *Microvascular research* 2002, 63(3):316-326.

192. Hattori M, Fujiyama A, Taylor TD, Watanabe H, Yada T, Park HS, Toyoda A, Ishii K, Totoki Y, Choi DK, Soeda E, Ohki M, Takagi T, Sakaki Y, Taudien S, Blechschmidt K, Polley A, Menzel U, Delabar J, Kumpf K, Lehmann R, Patterson D,

Reichwald K, Rump A, Schillhabel M, Schudy A, Zimmermann W, Rosenthal A, Kudoh J, Shibuya K, Kawasaki K, Asakawa S, Shintani A, Sasaki T, Nagamine K, Mitsuyama S, Antonarakis SE, Minoshima S, Shimizu N, Nordsiek G, Hornischer K, Brandt P, Scharfe M, Schon O, Desario A, Reichelt J, Kauer G, Blocker H, Ramser J, Beck A, Klages S, Hennig S, Riesselmann L, Dagand E, Haaf T, Wehrmeyer S, Borzym K, Gardiner K, Nizetic D, Francis F, Lehrach H, Reinhardt R, Yaspo ML. The DNA sequence of human chromosome 21. *Nature* 2000, 405(6784):311-319.

193. Hayflick L. Mortality and immortality at the cellular level. A review. *Biochemistry (Mosc)* 1997, 62(11):1180-1190.

194. Hébert CA, Baker JB. Interleukin-8: a review. *Cancer investigation* 1993, 11(6):743-750.

195. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome research* 1996, 6(10):986-994.

196. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, Otterson MF, Ota DM, Lugering N, Domschke W, Binion DG. Angiogenic Effects of Interleukin 8 (CXCL8) in Human Intestinal Microvascular Endothelial Cells Are Mediated by CXCR2. *Journal of Biological Chemistry* 2003, 278(10):8508-8515.

197. Hennighausen L, Robinson GW. Signaling pathways in mammary gland development. *Developmental cell* 2001, 1(4):467-475.

198. Hilberg F, Roth GJ, Krssak M, Kautschitsch S, Sommergruber W, Tontsch-Grunt U, Garin-Chesa P, Bader G, Zoephel A, Quant J, Heckel A, Rettig WJ. BIBF 1120: triple angiokinase inhibitor with sustained receptor blockade and good antitumor efficacy. *Cancer research* 2008, 68(12):4774-4782.

199. Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. Multiple control of interleukin-8 gene expression. *Journal of leukocyte biology* 2002, 72(5):847-855.

200. Hoffmann W, Jagla W. Cell type specific expression of secretory TFF peptides: colocalization with mucins and synthesis in the brain. *International review of cytology* 2002, 213:147-181.

201. Hoffmann W. Trefoil factor family (TFF) peptides and chemokine receptors: a promising relationship. *Journal of medicinal chemistry* 2009, 52(21):6505-6510.

202. Hoffmann W. Trefoil factor family (TFF) peptides: regulators of mucosal regeneration and repair, and more. *Peptides* 2004, 25(5):727-730.

203. Hoffmann W. TFF (trefoil factor family) peptides and their potential roles for differentiation processes during airway remodeling. *Current medicinal chemistry* 2007, 14(25):2716-2719.

204. Hoffmann W. TFF (Trefoil Factor Family) Peptides. In: *Handbook of Biologically Active Peptides*. Kastin AJ, eds. Burlington: Academic Press, 2006: pp 1147-1154.

205. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science (New York, NY)* 1999, 284(5422):1994-1998.

206. Holmes D, Zachary I. The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. *Genome Biology* 2005, 6(2):209.

207. Holmes WE, Lee J, Kuang WJ, Rice GC, Wood WI. Structure and functional expression of a human interleukin-8 receptor. *Science (New York, NY)* 1991, 253(5025):1278-1280.

208. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med* 1995, 1(2):149-153.

209. Horcher M, Rot A, Aschauer H, Besemer J. IL-8 derivatives with a reduced potential to form homodimers are fully active in vitro and in vivo. *Cytokine* 1998, 10(1):1-12.

210. Horvath CM, Wen Z, Darnell JE, Jr. A STAT protein domain that determines DNA sequence recognition suggests a novel DNA-binding domain. *Genes & development* 1995, 9(8):984-994.

211. Hovey R, Trott J, Vonderhaar B. Establishing a Framework for the Functional Mammary Gland: From Endocrinology to Morphology. *Journal of mammary gland biology and neoplasia* 2002, 7(1):17-38.

212. Howlader N, Noone A, Krapcho M, Garshell J, Neyman N, Altekruse S, Kosary C, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis D, Chen H, Feuer E, Cronin K. SEER Cancer Statistics Review, 1975-2010, National Cancer Institute. Bethesda, MD, . *SEER* 2014.

213. Hu DE, Hori Y, Fan TP. Interleukin-8 stimulates angiogenesis in rats. *Inflammation* 1993, 17(2):135-143.

214. Huang S, Mills L, Mian B, Tellez C, McCarty M, Yang XD, Gudas JM, Bar-Eli M. Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma. *The American journal of pathology* 2002, 161(1):125-134.

215. Huang Z, Bao SD. Roles of main pro- and anti-angiogenic factors in tumor angiogenesis. *World J Gastroenterol* 2004, 10(4):463-470.

216. Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res* 2000a, 6(5):2104-2119.

217. Inoue K, Slaton JW, Kim SJ, Perrotte P, Eve BY, Bar-Eli M, Radinsky R, Dinney CP. Interleukin 8 expression regulates tumorigenicity and metastasis in human bladder cancer. *Cancer research* 2000b, 60(8):2290-2299.

218. Inoue M, Hager JH, Ferrara N, Gerber HP, Hanahan D. VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. *Cancer Cell* 2002, 1(2):193-202.

219. Ito K, Ryuto M, Ushiro S, Ono M, Sugenoya A, Kuraoka A, Shibata Y, Kuwano M. Expression of tissue-type plasminogen activator and its inhibitor couples with

development of capillary network by human microvascular endothelial cells on Matrigel. *Journal of cellular physiology* 1995, 162(2):213-224.

220. Jacoby RF, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, Lubet RA, Kelloff GJ, Verma A, Moser AR, Dove WF. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. *Cancer research* 1996, 56(4):710-714.

221. Jagla W, Wiede A, Hinz M, Dietzmann K, Gulicher D, Gerlach KL, Hoffmann W. Secretion of TFF-peptides by human salivary glands. *Cell and tissue research* 1999, 298(1):161-166.

222. Jagla W, Wiede A, Dietzmann K, Rutkowski K, Hoffmann W. Co-localization of TFF3 peptide and oxytocin in the human hypothalamus. *Faseb j* 2000, 14(9):1126-1131.

223. Jain RK. Transport of molecules in the tumor interstitium: a review. *Cancer research* 1987, 47(12):3039-3051.

224. Jain RK. Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 2001, 7(9):987-989.

225. Jakowlew SB, Breathnach R, Jeltsch JM, Masiakowski P, Chambon P. Sequence of the pS2 mRNA induced by estrogen in the human breast cancer cell line MCF-7. *Nucleic acids research* 1984, 12(6):2861-2878.

226. Javed A, Lteif A. Development of the Human Breast. *Seminars in Plastic Surgery* 2013, 27(01):005-012.

227. Jiang G-x, Zhong X-y, Cui Y-f, Liu W, Tai S, Wang Z-d, Shi Y-g, Zhao S-y, Li C-l. IL-6/STAT3/TFF3 signaling regulates human biliary epithelial cell migration and wound healing in vitro. *Mol Biol Rep* 2010, 37(8):3813-3818.

228. Johnston SR. Clinical efforts to combine endocrine agents with targeted therapies against epidermal growth factor receptor/human epidermal growth factor receptor 2 and mammalian target of rapamycin in breast cancer. *Clin Cancer Res* 2006, 12(3 Pt 2):1061s-1068s.

229. Jones MK, Sarfeh IJ, Tarnawski AS. Induction of in vitro angiogenesis in the endothelial-derived cell line, EA hy926, by ethanol is mediated through PKC and MAPK. *Biochem Biophys Res Commun* 1998, 249(1):118-123.

230. Jordan VC, Gapstur S, Morrow M. Selective Estrogen Receptor Modulation and Reduction in Risk of Breast Cancer, Osteoporosis, and Coronary Heart Disease. *Journal of the National Cancer Institute* 2001, 93(19):1449-1457.

231. Jørgensen KH, Thim L, Jacobsen HE. Pancreatic spasmolytic polypeptide (PSP): I. Preparation and initial chemical characterization of a new polypeptide from porcine pancreas. *Regul Pept* 1982, 3(3-4):207-219.

232. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, Stingl J, Waterhouse PD, Khokha R. Progesterone induces adult mammary stem cell expansion. *Nature* 2010, 465(7299):803-807.

233. Jove R. Preface: STAT signaling. *Oncogene* 2000, 19(21):2466-2467.

234. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer* 2009, 9(4):239-252.

235. Kahn HJ, Marks A. A new monoclonal antibody, D2-40, for detection of lymphatic invasion in primary tumors. *Lab Invest* 2002, 82(9):1255-1257.

236. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006, 6(5):392-401.

237. Kanagarajadurai K, Sowdhamini R. Sequence and structural analyses of interleukin-8-like chemokine superfamily. *In silico biology* 2008, 8(3-4):307-330.

238. Kanai M, Mullen C, Podolsky DK. Intestinal trefoil factor induces inactivation of extracellular signal-regulated protein kinase in intestinal epithelial cells. *Proc Natl Acad Sci U S A* 1998, 95(1):178-182.

239. Kanayama H, Yano S, Kim SJ, Ozawa S, Ellis LM, Fidler IJ. Expression of vascular endothelial growth factor by human renal cancer cells enhances angiogenesis of primary tumors and production of ascites but not metastasis to the lungs in nude mice. *Clin Exp Metastasis* 1999, 17(10):831-840.

240. Kanda N, Watanabe S. 17beta-estradiol, progesterone, and dihydrotestosterone suppress the growth of human melanoma by inhibiting interleukin-8 production. *The Journal of investigative dermatology* 2001, 117(2):274-283.

241. Kannan N, Kang J, Kong X, Tang J, Perry JK, Mohankumar KM, Miller LD, Liu ET, Mertani HC, Zhu T, Grandison PM, Liu DX, Lobie PE. Trefoil factor 3 is oncogenic and mediates anti-estrogen resistance in human mammary carcinoma. *Neoplasia* 2010, 12(12):1041-1053.

242. Kansra S, Yamagata S, Sneade L, Foster L, Ben-Jonathan N. Differential effects of estrogen receptor antagonists on pituitary lactotroph proliferation and prolactin release. *Mol Cell Endocrinol* 2005, 239(1-2):27-36.

243. Karashima T, Sweeney P, Kamat A, Huang S, Kim SJ, Bar-Eli M, McConkey DJ, Dinney CP. Nuclear factor-kappaB mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8. *Clin Cancer Res* 2003, 9(7):2786-2797.

244. Kashima L, Toyota M, Mita H, Suzuki H, Idogawa M, Ogi K, Sasaki Y, Tokino T. CHFR, a potential tumor suppressor, downregulates interleukin-8 through the inhibition of NF-kappaB. *Oncogene* 2009, 28(29):2643-2653.

245. Kaulsay KK, Zhu T, Bennett WF, Lee K-O, Lobie PE. The Effects of Autocrine Human Growth Hormone (hGH) on Human Mammary Carcinoma Cell Behavior Are Mediated via the hGH Receptor. *Endocrinology* 2001, 142(2):767-777.

246. Kerbel RS, Klement G, Pritchard KI, Kamen B. Continuous low-dose anti-angiogenic/ metronomic chemotherapy: from the research laboratory into the oncology clinic. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 2002, 13(1):12-15.

247. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. *The lancet oncology* 2001, 2(3):133-140.

248. Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, Brenner HJ. Establishment and characterization of a cell line of human breast carcinoma origin. *Eur J Cancer* 1979, 15(5):659-670.

249. Khamis ZI, Sahab ZJ, Sang QX. Active roles of tumor stroma in breast cancer metastasis. *Int J Breast Cancer* 2012, 2012:574025.

250. Khan N, Afaq F, Mukhtar H. Lifestyle as risk factor for cancer: Evidence from human studies. *Cancer Letters* 2010, 293(2):133-143.

251. Kim S-W, Kim H, Cho H-J, Lee J-U, Levit R, Yoon Y-s. Human Peripheral Blood-Derived CD31+ Cells Have Robust Angiogenic and Vasculogenic Properties and Are Effective for Treating Ischemic Vascular Disease. *Journal of the American College of Cardiology* 2010, 56(7):593-607.

252. Kindon H, Pothoulakis C, Thim L, Lynch-Devaney K, Podolsky DK. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. *Gastroenterology* 1995, 109(2):516-523.

253. Kinoshita K, Taupin DR, Itoh H, Podolsky DK. Distinct pathways of cell migration and antiapoptotic response to epithelial injury: structure-function analysis of human intestinal trefoil factor. *Mol Cell Biol* 2000a, 20(13):4680-4690.

254. Kinoshita K, Taupin DR, Itoh H, Podolsky DK. Distinct Pathways of Cell Migration and Antiapoptotic Response to Epithelial Injury: Structure-Function Analysis of Human Intestinal Trefoil Factor. *Molecular and Cellular Biology* 2000b, 20(13):4680-4690.

255. Kitadai Y, Takahashi Y, Haruma K, Naka K, Sumii K, Yokozaki H, Yasui W, Mukaida N, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice. *Br J Cancer* 1999, 81(4):647-653.

256. Kitadai Y, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *The American journal of pathology* 1998, 152(1):93-100.

257. Kjellev S. The trefoil factor family - small peptides with multiple functionalities. *Cell Mol Life Sci* 2009, 66(8):1350-1369.

258. Klagsbrun M, D'Amore PA. Regulators of angiogenesis. *Annual review of physiology* 1991, 53:217-239.

259. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science (New York, NY)* 1992, 258(5089):1798-1801.

260. Korohoda W, Madeja Z. Contact of sarcoma cells with aligned fibroblasts accelerates their displacement: computer-assisted analysis of tumour cell locomotion in co-

culture. *Biochemistry and cell biology = Biochimie et biologie cellulaire* 1997, 75(3):263-276.

261. Kraljevic Pavelic S, Sedic M, Bosnjak H, Spaventi S, Pavelic K. Metastasis: new perspectives on an old problem. *Mol Cancer* 2011, 10:22.

262. Kurokawa H, Lenferink AE, Simpson JF, Pisacane PI, Sliwkowski MX, Forbes JT, Arteaga CL. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer research* 2000, 60(20):5887-5894.

263. Kurokawa H, Arteaga CL. ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms. *Clin Cancer Res* 2003, 9(1 Pt 2):511s-515s.

264. Labayle D, Fischer D, Vielh P, Drouhin F, Pariente A, Bories C, Duhamel O, Trousset M, Attali P. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterology* 1991, 101(3):635-639.

265. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat* 2004, 83(3):249-289.

266. Lacroix M. Significance, detection and markers of disseminated breast cancer cells. *Endocr Relat Cancer* 2006, 13(4):1033-1067.

267. Lakshminarayanan V, Drab-Weiss EA, Roebuck KA. H2O2 and tumor necrosis factor-alpha induce differential binding of the redox-responsive transcription factors AP-1 and NF-kappaB to the interleukin-8 promoter in endothelial and epithelial cells. *J Biol Chem* 1998, 273(49):32670-32678.

268. Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. *Circulation research* 2007, 100(6):782-794.

269. Lange CA, Yee D. Progesterone and breast cancer. *Women's health (London, England)* 2008, 4(2):151-162.

270. Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene* 2005, 24(50):7443-7454.

271. Law ML, Kao FT, Wei Q, Hartz JA, Greene GL, Zarucki-Schulz T, Conneely OM, Jones C, Puck TT, O'Malley BW, et al. The progesterone receptor gene maps to human chromosome band 11q13, the site of the mammary oncogene int-2. *Proc Natl Acad Sci U S A* 1987, 84(9):2877-2881.

272. Lawler J. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *Journal of cellular and molecular medicine* 2002, 6(1):1-12.

273. Le X, Shi Q, Wang B, Xiong Q, Qian C, Peng Z, Li XC, Tang H, Abbruzzese JL, Xie K. Molecular regulation of constitutive expression of interleukin-8 in human pancreatic adenocarcinoma. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 2000, 20(11):935-946.

274. Lebrun J-J. The Dual Role of TGF in Human Cancer: From Tumor Suppression to Cancer Metastasis. *ISRN Molecular Biology* 2012, 2012:28.

275. Lee S, Kolonel L, Wilkens L, Wan P, Henderson B, Pike M. Postmenopausal hormone therapy and breast cancer risk: the Multiethnic Cohort. *International journal of cancer Journal international du cancer* 2006, 118(5):1285-1291.

276. Lee YS, Choi I, Ning Y, Kim NY, Khatchadourian V, Yang D, Chung HK, Choi D, LaBonte MJ, Ladner RD, Nagulapalli Venkata KC, Rosenberg DO, Petasis NA, Lenz HJ, Hong YK. Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis. *Br J Cancer* 2012, 106(11):1833-1841.

277. Lefebvre O, Chenard MP, Masson R, Linares J, Dierich A, LeMeur M, Wendling C, Tomasetto C, Chambon P, Rio MC. Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. *Science (New York, NY)* 1996, 274(5285):259-262.

278. Lei Y-K, You Z-H, Ji Z, Zhu L, Huang D-S. Assessing and predicting protein interactions by combining manifold embedding with multiple information integration. *BMC Bioinformatics* 2012, 13(Suppl 7):S3.

279. Leibovici J, Itzhaki O, Huszar M, Sinai J. The tumor microenvironment: part 1. *Immunotherapy* 2011, 3(11):1367-1384.

280. Lester S. Perspectives on margins in DCIS: pathology. *Journal of the National Comprehensive Cancer Network : JNCCN* 2010, 8(10):1219-1222.

281. Li A, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis* 2005, 8(1):63-71.

282. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol* 2003a, 170(6):3369-3376.

283. Li J, Zhou R, He WC, Xia B. Effects of recombinant human intestinal trefoil factor on trinitrobenzene sulphonic acid induced colitis in rats. *Mol Biol Rep* 2011, 38(7):4787-4792.

284. Li X, Fu GF, Fan YR, Shi CF, Liu XJ, Xu GX, Wang JJ. Potent inhibition of angiogenesis and liver tumor growth by administration of an aerosol containing a transferrin-liposome-endostatin complex. *World J Gastroenterol* 2003b, 9(2):262-266.

285. Lin Y, Huang R, Chen L, Li S, Shi Q, Jordan C, Huang RP. Identification of interleukin-8 as estrogen receptor-regulated factor involved in breast cancer invasion and angiogenesis by protein arrays. *International journal of cancer Journal international du cancer* 2004, 109(4):507-515.

286. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001, 411(6835):375-379.

287. Liu D, el-Hariry I, Karayiannakis AJ, Wilding J, Chinery R, Kmiot W, McCrea PD, Gullick WJ, Pignatelli M. Phosphorylation of beta-catenin and epidermal growth factor receptor by intestinal trefoil factor. *Lab Invest* 1997, 77(6):557-563.

288. Loncar MB, Al-azzeh ED, Sommer PS, Marinovic M, Schmehl K, Kruschewski M, Blin N, Stohwasser R, Gott P, Kayademir T. Tumour necrosis factor alpha and nuclear factor kappaB inhibit transcription of human TFF3 encoding a gastrointestinal healing peptide. *Gut* 2003, 52(9):1297-1303.

289. Longman RJ, Douthwaite J, Sylvester PA, Poulsom R, Corfield AP, Thomas MG, Wright NA. Coordinated localisation of mucins and trefoil peptides in the ulcer associated cell lineage and the gastrointestinal mucosa. *Gut* 2000, 47(6):792-800.

290. Lowman HB, Fairbrother WJ, Slagle PH, Kabakoff R, Liu J, Shire S, Hebert CA. Monomeric variants of IL-8: effects of side chain substitutions and solution conditions upon dimer formation. *Protein science : a publication of the Protein Society* 1997, 6(3):598-608.

291. Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS. Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. *Lung cancer (Amsterdam, Netherlands)* 2007, 56(1):25-33.

292. Macdonald FCAGFCHJ. Molecular biology of cancer. London: BIOS Scientific; 2004.

293. Macias H, Hinck L. Mammary gland development. *Wiley interdisciplinary reviews Developmental biology* 2012, 1(4):533-557.

294. Mack MG, Straub R, Eichler K, Sollner O, Lehnert T, Vogl TJ. Breast cancer metastases in liver: laser-induced interstitial thermotherapy--local tumor control rate and survival data. *Radiology* 2004, 233(2):400-409.

295. Majure J. Breast Cancer: Enslow; 2000.

296. Man YG, Sang QX. The significance of focal myoepithelial cell layer disruptions in human breast tumor invasion: a paradigm shift from the "protease-centered" hypothesis. *Experimental cell research* 2004, 301(2):103-118.

297. Man YG. Focal degeneration of aged or injured myoepithelial cells and the resultant auto-immunoreactions are trigger factors for breast tumor invasion. *Medical hypotheses* 2007, 69(6):1340-1357.

298. Marchbank T, Westley BR, May FE, Calnan DP, Playford RJ. Dimerization of human pS2 (TFF1) plays a key role in its protective/healing effects. *The Journal of pathology* 1998, 185(2):153-158.

299. Martin D, Galisteo R, Gutkind JS. CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. *J Biol Chem* 2009, 284(10):6038-6042.

300. Martin V, Ribieras S, Song-Wang XG, Lasne Y, Frappart L, Rio MC, Dante R. Involvement of DNA methylation in the control of the expression of an estrogen-

induced breast-cancer-associated protein (pS2) in human breast cancers. *Journal of cellular biochemistry* 1997, 65(1):95-106.

301. Mashimo H, Wu DC, Podolsky DK, Fishman MC. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. *Science (New York, NY)* 1996, 274(5285):262-265.

302. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic acids research* 1982, 10(24):7895-7903.

303. Masters JR. Human cancer cell lines: fact and fantasy. *Nat Rev Mol Cell Biol* 2000, 1(3):233-236.

304. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S. Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci U S A* 1993, 90(21):10193-10197.

305. May FE, Church ST, Major S, Westley BR. The closely related estrogen-regulated trefoil proteins TFF1 and TFF3 have markedly different hydrodynamic properties, overall charge, and distribution of surface charge. *Biochemistry* 2003, 42(27):8250-8259.

306. May FE, Westley BR. Expression of human intestinal trefoil factor in malignant cells and its regulation by oestrogen in breast cancer cells. *The Journal of pathology* 1997a, 182(4):404-413.

307. May FE, Westley BR. Trefoil proteins: their role in normal and malignant cells. *The Journal of pathology* 1997b, 183(1):4-7.

308. May FEB, Griffin SM, Westley BR. The trefoil factor interacting protein TFIZ1 binds the trefoil protein TFF1 preferentially in normal gastric mucosal cells but the co-expression of these proteins is deregulated in gastric cancer. *The International Journal of Biochemistry & Cell Biology* 2009, 41(3):632-640.

309. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 2006, 15(6):1159-1169.

310. McKenzie C, Marchbank T, Playford RJ, Otto W, Thim L, Parsons ME. Pancreatic spasmolytic polypeptide protects the gastric mucosa but does not inhibit acid secretion or motility. *The American journal of physiology* 1997, 273(1 Pt 1):G112-117.

311. McKenzie C, Thim L, Parsons ME. Topical and intravenous administration of trefoil factors protect the gastric mucosa from ethanol-induced injury in the rat. *Alimentary pharmacology & therapeutics* 2000, 14(8):1033-1040.

312. Medina D. The mammary gland: a unique organ for the study of development and tumorigenesis. *Journal of mammary gland biology and neoplasia* 1996, 1(1):5-19.

313. Merritt WM, Lin YG, Spannuth WA, Fletcher MS, Kamat AA, Han LY, Landen CN, Jennings N, De Geest K, Langley RR, Villares G, Sanguino A, Lutgendorf SK, Lopez-Berestein G, Bar-Eli MM, Sood AK. Effect of interleukin-8 gene silencing with liposome-encapsulated small interfering RNA on ovarian cancer cell growth. *J Natl Cancer Inst* 2008, 100(5):359-372.

314. Metzger-Filho O, Winer EP, Krop I. Pertuzumab: optimizing HER2 blockade. *Clin Cancer Res* 2013, 19(20):5552-5556.

315. Meyer zum Buschenfelde D, Hoschutzky H, Tauber R, Huber O. Molecular mechanisms involved in TFF3 peptide-mediated modulation of the E-cadherin/catenin cell adhesion complex. *Peptides* 2004, 25(5):873-883.

316. Mian BM, Dinney CP, Bermejo CE, Sweeney P, Tellez C, Yang XD, Gudas JM, McConkey DJ, Bar-Eli M. Fully human anti-interleukin 8 antibody inhibits tumor growth in orthotopic bladder cancer xenografts via down-regulation of matrix metalloproteases and nuclear factor-kappaB. *Clin Cancer Res* 2003, 9(8):3167-3175.

317. Mikhitarian K, Gillanders WE, Almeida JS, Hebert Martin R, Varela JC, Metcalf JS, Cole DJ, Mitas M. An innovative microarray strategy identities informative molecular markers for the detection of micrometastatic breast cancer. *Clin Cancer Res* 2005, 11(10):3697-3704.

318. Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET, Bergh J. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci U S A* 2005, 102(38):13550-13555.

319. Miller LJ, Kurtzman SH, Wang Y, Anderson KH, Lindquist RR, Kreutzer DL. Expression of interleukin-8 receptors on tumor cells and vascular endothelial cells in human breast cancer tissue. *Anticancer Res* 1998, 18(1A):77-81.

320. Miller MW, Nowakowski RS. Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. *Brain research* 1988, 457(1):44-52.

321. Minegishi Y, Suzuki H, Arakawa M, Fukushima Y, Masaoka T, Ishikawa T, Wright NA, Hibi T. Reduced Shh expression in TFF2-overexpressing lesions of the gastric fundus under hypochlorhydric conditions. *The Journal of pathology* 2007, 213(2):161-169.

322. Monnerat C, Chompret A, Kannengiesser C, Avril MF, Janin N, Spatz A, Guinebretiere JM, Marian C, Barrois M, Boitier F, Lenoir GM, Bressac-de Paillerets B. BRCA1, BRCA2, TP53, and CDKN2A germline mutations in patients with breast cancer and cutaneous melanoma. *Fam Cancer* 2007, 6(4):453-461.

323. Morabia A, Abel T. The WHO report "Preventing chronic diseases: a vital investment" and us. *Sozial- und Praventivmedizin* 2006, 51(2):74.

324. Moses MA. The regulation of neovascularization of matrix metalloproteinases and their inhibitors. *Stem cells (Dayton, Ohio)* 1997, 15(3):180-189.

325. Mukaida N, Okamoto S, Ishikawa Y, Matsushima K. Molecular mechanism of interleukin-8 gene expression. *Journal of leukocyte biology* 1994, 56(5):554-558.

326. Mukaida N, Mahe Y, Matsushima K. Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. *J Biol Chem* 1990, 265(34):21128-21133.

327. Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol* 1989, 143(4):1366-1371.

328. Mulac-Jericevic B, Lydon JP, DeMayo FJ, Conneely OM. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proc Natl Acad Sci U S A* 2003, 100(17):9744-9749.

329. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001, 410(6824):50-56.

330. Mulugeta S, Nguyen V, Russo SJ, Muniswamy M, Beers MF. A surfactant protein C precursor protein BRICHOS domain mutation causes endoplasmic reticulum stress, proteasome dysfunction, and caspase 3 activation. *American journal of respiratory cell and molecular biology* 2005, 32(6):521-530.

331. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005, 11(2 Pt 2):937s-943s.

332. Murdoch C, Monk PN, Finn A. Cxc chemokine receptor expression on human endothelial cells. *Cytokine* 1999, 11(9):704-712.

333. Murphy PM, Tiffany HL. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science (New York, NY)* 1991, 253(5025):1280-1283.

334. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med* 2002, 196(11):1497-1506.

335. Nastase A, Paslaru L, Niculescu AM, Ionescu M, Dumitrascu T, Herlea V, Dima S, Gheorghe C, Lazar V, Popescu I. Prognostic and predictive potential molecular biomarkers in colon cancer. *Chirurgia (Bucharest, Romania : 1990)* 2011, 106(2):177-185.

336. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 2010, 11(3):220-228.

337. Nicholson RI, Hutcheson IR, Jones HE, Hiscox SE, Giles M, Taylor KM, Gee JM. Growth factor signalling in endocrine and anti-growth factor resistant breast cancer. *Reviews in endocrine & metabolic disorders* 2007, 8(3):241-253.

338. Ning Y, Manegold PC, Hong YK, Zhang W, Pohl A, Lurje G, Winder T, Yang D, LaBonte MJ, Wilson PM, Ladner RD, Lenz HJ. Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. *International journal of cancer Journal international du cancer* 2011, 128(9):2038-2049.

339. Ning Y, Manegold PC, Hong YK, Zhang W, Pohl A, Lurje G, Winder T, Yang D, LaBonte MJ, Wilson PM, Ladner RD, Lenz H-J. Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. *International Journal of Cancer* 2010, 128(9):2038-2049.

340. Nishi M, Abe Y, Tomii Y, Tsukamoto H, Kijima H, Yamazaki H, Ohnishi Y, Iwasaki M, Inoue H, Ueyama Y, Nakamura M. Cell binding isoforms of vascular endothelial growth factor-A (VEGF189) contribute to blood flow-distant metastasis of pulmonary adenocarcinoma. *Int J Oncol* 2005, 26(6):1517-1524.

341. Niu G, Heller R, Catlett-Falcone R, Coppola D, Jaroszeski M, Dalton W, Jove R, Yu H. Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo. *Cancer research* 1999, 59(20):5059-5063.

342. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, Zhang S, Wang T, Sinibaldi D, Coppola D, Heller R, Ellis LM, Karras J, Bromberg J, Pardoll D, Jove R, Yu H. Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 2002, 21(13):2000-2008.

343. Nor JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *The American journal of pathology* 1999, 154(2):375-384.

344. Normanno N, Di Maio M, De Maio E, De Luca A, de Matteis A, Giordano A, Perrone F. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr Relat Cancer* 2005, 12(4):721-747.

345. North S, Moenner M, Bikfalvi A. Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. *Cancer Letters* 2005, 218(1):1-14.

346. Nunes RA, Harris LN. The HER2 extracellular domain as a prognostic and predictive factor in breast cancer. *Clinical breast cancer* 2002, 3(2):125-135; discussion 136-127.

347. Nunez AM, Berry M, Imler JL, Chambon P. The 5' flanking region of the pS2 gene contains a complex enhancer region responsive to oestrogens, epidermal growth factor, a tumour promoter (TPA), the c-Ha-ras oncoprotein and the c-jun protein. *Embo j* 1989, 8(3):823-829.

348. Nunez AM, Jakowlev S, Briand JP, Gaire M, Krust A, Rio MC, Chambon P. Characterization of the estrogen-induced pS2 protein secreted by the human breast cancer cell line MCF-7. *Endocrinology* 1987, 121(5):1759-1765.

349. Nussenbaum F, Herman IM. Tumor angiogenesis: insights and innovations. *Journal of oncology* 2010, 2010:132641.

350. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994, 79(2):315-328.

351. Oertel M, Graness A, Thim L, Buhling F, Kalbacher H, Hoffmann W. Trefoil factor family-peptides promote migration of human bronchial epithelial cells: synergistic effect with epidermal growth factor. *American journal of respiratory cell and molecular biology* 2001, 25(4):418-424.

352. Oka M, Sakaguchi M, Okada T, Nagai H, Ozaki M, Yoshioka T, Inoue H, Mukaida N, Kikkawa U, Nishigori C. Signal transducer and activator of transcription 3 upregulates interleukin-8 expression at the level of transcription in human melanoma cells. *Experimental dermatology* 2010, 19(8):e50-55.

353. Okada H, Kimura MT, Tan D, Fujiwara K, Igarashi J, Makuuchi M, Hui AM, Tsurumaru M, Nagase H. Frequent trefoil factor 3 (TFF3) overexpression and promoter hypomethylation in mouse and human hepatocellular carcinomas. *Int J Oncol* 2005, 26(2):369-377.

354. Okamura K, Morimoto A, Hamanaka R, Ono M, Kohno K, Uchida Y, Kuwano M. A model system for tumor angiogenesis: involvement of transforming growth factor-alpha in tube formation of human microvascular endothelial cells induced by esophageal cancer cells. *Biochem Biophys Res Commun* 1992, 186(3):1471-1479.

355. Olayioye MA. Update on HER-2 as a target for cancer therapy: intracellular signaling pathways of ErbB2/HER-2 and family members. *Breast Cancer Res* 2001, 3(6):385-389.

356. Olumi AF, Dazin P, Tlsty TD. A novel coculture technique demonstrates that normal human prostatic fibroblasts contribute to tumor formation of LNCaP cells by retarding cell death. *Cancer research* 1998, 58(20):4525-4530.

357. Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, Kelly PA. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes & development* 1997, 11(2):167-178.

358. Ormandy CJ, Naylor M, Harris J, Robertson F, Horseman ND, Lindeman GJ, Visvader J, Kelly PA. Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice. *Recent progress in hormone research* 2003, 58:297-323.

359. Osborne C, Wilson P, Tripathy D. Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. *Oncologist* 2004, 9(4):361-377.

360. Osborne MP. Breast cancer prevention by antiestrogens. *Annals of the New York Academy of Sciences* 1999, 889:146-151.

361. Oseni T, Patel R, Pyle J, Jordan VC. Selective estrogen receptor modulators and phytoestrogens. *Planta medica* 2008, 74(13):1656-1665.

362. Osugi T, Oshima Y, Fujio Y, Funamoto M, Yamashita A, Negoro S, Kunisada K, Izumi M, Nakaoka Y, Hirota H, Okabe M, Yamauchi-Takihara K, Kawase I, Kishimoto T. Cardiac-specific activation of signal transducer and activator of transcription 3 promotes vascular formation in the heart. *The Journal of biological chemistry* 2002, 277(8):6676-6681.

363. Otto WR, Thim L. Trefoil factor family-interacting proteins. *Cell Mol Life Sci* 2005, 62(24):2939-2946.

364. Oude Nijhuis CS, Vellenga E, Daenen SM, Kamps WA, De Bont ES. Endothelial cells are main producers of interleukin 8 through Toll-like receptor 2 and 4 signaling during bacterial infection in leukopenic cancer patients. *Clinical and diagnostic laboratory immunology* 2003, 10(4):558-563.

365. Owens MB, Hill AD, Hopkins AM. Ductal barriers in mammary epithelium. *Tissue barriers* 2013, 1(4):e25933.

366. Ozgul C, Karaoz E, Erdogan D, Dursun A. Expression of epidermal growth factor receptor in normal colonic mucosa and in adenocarcinomas of the colon. *Acta physiologica Hungarica* 1997, 85(2):121-128.

367. Pace P, Taylor J, Suntharalingam S, Coombes RC, Ali S. Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha. *J Biol Chem* 1997, 272(41):25832-25838.

368. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Casanovas O. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009, 15(3):220-231.

369. Pandey V, Wu ZS, Zhang M, Li R, Zhang J, Zhu T, Lobie PE. Trefoil factor 3 promotes metastatic seeding and predicts poor survival outcome of patients with mammary carcinoma. *Breast Cancer Res* 2014, 16(5):429.

370. Papetti M, Herman IM. Mechanisms of normal and tumor-derived angiogenesis. *Am J Physiol Cell Physiol* 2002, 282(5):C947-970.

371. Parangi S, O'Reilly M, Christofori G, Holmgren L, Grosfeld J, Folkman J, Hanahan D. Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. *Proc Natl Acad Sci U S A* 1996, 93(5):2002-2007.

372. Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. *Molecular medicine today* 2000, 6(8):324-329.

373. Pasqualini JR. The selective estrogen enzyme modulators in breast cancer: a review. *Biochim Biophys Acta* 2004, 1654(2):123-143.

374. Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 1998, 392(6672):190-193.

375. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. *Nature* 2000, 406(6797):747-752.

376. Perry JK, Kannan N, Grandison PM, Mitchell MD, Lobie PE. Are trefoil factors oncogenic? *Trends Endocrinol Metab* 2008, 19(2):74-81.

377. Persson I. Estrogens in the causation of breast, endometrial and ovarian cancers - evidence and hypotheses from epidemiological findings. *The Journal of steroid biochemistry and molecular biology* 2000, 74(5):357-364.

378. Petreaca ML, Yao M, Liu Y, DeFea K, Martins-Green M. Transactivation of Vascular Endothelial Growth Factor Receptor-2 by Interleukin-8 (IL-8/CXCL8) Is Required for IL-8/CXCL8-induced Endothelial Permeability. *Molecular Biology of the Cell* 2007, 18(12):5014-5023.

379. Pharoah PD, Day NE, Duffy S, Easton DF, Ponder BA. Family history and the risk of breast cancer: a systematic review and meta-analysis. *International journal of cancer Journal international du cancer* 1997, 71(5):800-809.

380. Piggott NH, Henry JA, May FE, Westley BR. Antipeptide antibodies against the pNR-2 oestrogen-regulated protein of human breast cancer cells and detection of pNR-2 expression in normal tissues by immunohistochemistry. *The Journal of pathology* 1991, 163(2):95-104.

381. Playford RJ, Marchbank T, Chinery R, Evison R, Pignatelli M, Boulton RA, Thim L, Hanby AM. Human spasmolytic polypeptide is a cytoprotective agent that stimulates cell migration. *Gastroenterology* 1995, 108(1):108-116.

382. Podolsky DK. Mechanisms of regulatory peptide action in the gastrointestinal tract: trefoil peptides. *Journal of gastroenterology* 2000, 35 Suppl 12:69-74.

383. Polshakov VI, Williams MA, Gargaro AR, Frenkiel TA, Westley BR, Chadwick MP, May FE, Feeney J. High-resolution solution structure of human pNR-2/pS2: a single trefoil motif protein. *Journal of molecular biology* 1997, 267(2):418-432.

384. Polverini PJ. The pathophysiology of angiogenesis. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists* 1995, 6(3):230-247.

385. Polyak K, Kalluri R. The role of the microenvironment in mammary gland development and cancer. *Cold Spring Harbor perspectives in biology* 2010, 2(11):a003244.

386. Polyak K. Breast cancer: origins and evolution. *The Journal of Clinical Investigation* 2007, 117(11):3155-3163.

387. Poste G, Fidler IJ. The pathogenesis of cancer metastasis. *Nature* 1980, 283(5743):139-146.

388. Poulsom R. Trefoil peptides. *Bailliere's clinical gastroenterology* 1996, 10(1):113-134.

389. Poulsom R, Hanby AM, Lalani EN, Hauser F, Hoffmann W, Stamp GW. Intestinal trefoil factor (TFF 3) and pS2 (TFF 1), but not spasmolytic polypeptide (TFF 2) mRNAs are co-expressed in normal, hyperplastic, and neoplastic human breast epithelium. *The Journal of pathology* 1997, 183(1):30-38.

390. Prest SJ, May FE, Westley BR. The estrogen-regulated protein, TFF1, stimulates migration of human breast cancer cells. *Faseb j* 2002, 16(6):592-594.

391. Probst JC, Zetzsche T, Weber M, Theilemann P, Skutella T, Landgraf R, Jirikowski GF. Human intestinal trefoil factor is expressed in human hypothalamus and pituitary: evidence for a novel neuropeptide. *Faseb j* 1996, 10(13):1518-1523.

392. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 2003, 9(6):677-684.

393. Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D, Bucana CD, Fidler IJ. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin Cancer Res* 1995, 1(1):19-31.

394. Raina V. Is fulvestrant more effective than tamoxifen for treating ER-positive breast cancer in postmenopausal women? *Nat Clin Pract Oncol* 2004, 1(1):20-21.

395. Rajarathnam K, Sykes BD, Kay CM, Dewald B, Geiser T, Baggiolini M, Clark-Lewis I. Neutrophil activation by monomeric interleukin-8. *Science (New York, NY)* 1994, 264(5155):90-92.

396. Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R, Ruoslahti E. Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. *J Clin Invest* 1998, 102(2):430-437.

397. Ramjeesingh R, Leung R, Siu CH. Interleukin-8 secreted by endothelial cells induces chemotaxis of melanoma cells through the chemokine receptor CXCR1. *Faseb j* 2003, 17(10):1292-1294.

398. Ramsay DT, Kent JC, Hartmann RA, Hartmann PE. Anatomy of the lactating human breast redefined with ultrasound imaging. *Journal of anatomy* 2005, 206(6):525-534.

399. Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology : the official journal of the International Society for Pathophysiology / ISP* 2010, 17(4):229-251.

400. Reeves GK, Pirie K, Beral V, Green J, Spencer E, Bull D. Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study. *BMJ* 2007, 335(7630):1134.

401. Regalo G, Wright NA, Machado JC. Trefoil factors: from ulceration to neoplasia. *Cell Mol Life Sci* 2005, 62(24):2910-2915.

402. Ribieras S, Tomasetto C, Rio M-C. The pS2/TFF1 trefoil factor, from basic research to clinical applications. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1998, 1378(1):F61-F77.

403. Ribieras S, Lefebvre O, Tomasetto C, Rio MC. Mouse Trefoil factor genes: genomic organization, sequences and methylation analyses. *Gene* 2001, 266(1-2):67-75.

404. Richards BL, Eisma RJ, Spiro JD, Lindquist RL, Kreutzer DL. Coexpression of interleukin-8 receptors in head and neck squamous cell carcinoma. *American journal of surgery* 1997, 174(5):507-512.

405. Richert M, Schwertfeger K, Ryder J, Anderson S. An Atlas of Mouse Mammary Gland Development. *Journal of mammary gland biology and neoplasia* 2000, 5(2):227-241.

406. Richmond A, Yang J, Su Y. The good and the bad of chemokines/chemokine receptors in melanoma. *Pigment Cell Melanoma Res* 2009, 22(2):175-186.

407. Rickman DS, Chen YB, Banerjee S, Pan Y, Yu J, Vuong T, Perner S, Lafargue CJ, Mertz KD, Setlur SR, Sircar K, Chinnaiyan AM, Bismar TA, Rubin MA, Demichelis F. ERG cooperates with androgen receptor in regulating trefoil factor 3 in prostate cancer disease progression. *Neoplasia* 2010, 12(12):1031-1040.

408. Rieger-Christ KM, Pezza JA, Dugan JM, Braasch JW, Hughes KS, Summerhayes IC. Disparate E-cadherin mutations in LCIS and associated invasive breast carcinomas. *Molecular pathology : MP* 2001, 54(2):91-97.

409. Rini BI, Small EJ. Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma. *J Clin Oncol* 2005, 23(5):1028-1043.

410. Rio MC, Bellocq JP, Gairard B, Rasmussen UB, Krust A, Koehl C, Calderoli H, Schiff V, Renaud R, Chambon P. Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. *Proc Natl Acad Sci U S A* 1987, 84(24):9243-9247.

411. Risau W. Mechanisms of angiogenesis. *Nature* 1997, 386(6626):671-674.

412. Rivat C, Rodrigues S, Bruyneel E, Pietu G, Robert A, Redeuilh G, Bracke M, Gespach C, Attoub S. Implication of STAT3 signaling in human colonic cancer cells during intestinal trefoil factor 3 (TFF3) -- and vascular endothelial growth factor-mediated cellular invasion and tumor growth. *Cancer research* 2005, 65(1):195-202.

413. Robinson CJ, Stringer SE. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *Journal of cell science* 2001, 114(Pt 5):853-865.

414. Rodrigues S, Nguyen QD, Faivre S, Bruyneel E, Thim L, Westley B, May F, Flatau G, Mareel M, Gespach C, Emami S. Activation of cellular invasion by trefoil peptides and src is mediated by cyclooxygenase- and thromboxane A2 receptor-dependent signaling pathways. *FASEB J* 2001, 15(9):1517-1528.

415. Rodrigues S, Attoub S, Nguyen QD, Bruyneel E, Rodrigue CM, Westley BR, May FE, Thim L, Mareel M, Emami S, Gespach C. Selective abrogation of the proinvasive activity of the trefoil peptides pS2 and spasmolytic polypeptide by disruption of the EGF receptor signaling pathways in kidney and colonic cancer cells. *Oncogene* 2003a, 22(29):4488-4497.

416. Rodrigues S, Van Aken E, Van Bocxlaer S, Attoub S, Nguyen QD, Bruyneel E, Westley BR, May FE, Thim L, Mareel M, Gespach C, Emami S. Trefoil peptides as proangiogenic factors in vivo and in vitro: implication of cyclooxygenase-2 and EGF receptor signaling. *FASEB J* 2003b, 17(1):7-16.

417. Rody A, Karn T, Liedtke C, Pusztai L, Ruckhaeberle E, Hanker L, Gaetje R, Solbach C, Ahr A, Metzler D, Schmidt M, Muller V, Holtrich U, Kaufmann M. A clinically relevant gene signature in triple negative and basal-like breast cancer. *Breast Cancer Res* 2011, 13(5):R97.

418. Roebuck KA. Regulation of interleukin-8 gene expression. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 1999, 19(5):429-438.

419. Roebuck KA, Carpenter LR, Lakshminarayanan V, Page SM, Moy JN, Thomas LL. Stimulus-specific regulation of chemokine expression involves differential activation of the redox-responsive transcription factors AP-1 and NF-kappaB. *Journal of leukocyte biology* 1999, 65(3):291-298.

420. Rollins BJ. Chemokines. *Blood* 1997, 90(3):909-928.

421. Ronnov-Jessen L, Bissell MJ. Breast cancer by proxy: can the microenvironment be both the cause and consequence? *Trends in molecular medicine* 2009, 15(1):5-13.

422. Rosa FE, Caldeira JR, Felipes J, Bertonha FB, Quevedo FC, Domingues MA, Moraes Neto FA, Rogatto SR. Evaluation of estrogen receptor alpha and beta and progesterone receptor expression and correlation with clinicopathologic factors and proliferative marker Ki-67 in breast cancers. *Hum Pathol* 2008, 39(5):720-730.

423. Ross JS, Fletcher JA. The HER-2/neu Oncogene in Breast Cancer: Prognostic Factor, Predictive Factor, and Target for Therapy. *Oncologist* 1998, 3(4):237-252.

424. Ruchaud-Sparagano MH, Westley BR, May FE. The trefoil protein TFF1 is bound to MUC5AC in human gastric mucosa. *Cell Mol Life Sci* 2004, 61(15):1946-1954.

425. Ruddon RW. Cancer Biology: Oxford University Press, USA; 2007.

426. Sager R. Expression genetics in cancer: shifting the focus from DNA to RNA. *Proc Natl Acad Sci U S A* 1997, 94(3):952-955.

427. Salcedo R, Resau JH, Halverson D, Hudson EA, Dambach M, Powell D, Wasserman K, Oppenheim JJ. Differential expression and responsiveness of chemokine receptors (CXCR1-3) by human microvascular endothelial cells and umbilical vein endothelial cells. *FASEB J* 2000a, 14(13):2055-2064.

428. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 2000b, 96(1):34-40.

429. Sands BE, Podolsky DK. The trefoil peptide family. *Annual review of physiology* 1996, 58:253-273.

430. Sariego J. Breast cancer in the young patient. *The American surgeon* 2010, 76(12):1397-1400.

431. Sasaki M, Ikeda H, Nakanuma Y. Expression profiles of MUC mucins and trefoil factor family (TFF) peptides in the intrahepatic biliary system: physiological distribution and pathological significance. *Progress in histochemistry and cytochemistry* 2007, 42(2):61-110.

432. Schadendorf D, Moller A, Algermissen B, Worm M, Sticherling M, Czarnetzki BM. IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. *J Immunol* 1993, 151(5):2667-2675.

433. Schaefer LK, Ren Z, Fuller GN, Schaefer TS. Constitutive activation of Stat3alpha in brain tumors: localization to tumor endothelial cells and activation by the endothelial tyrosine kinase receptor (VEGFR-2). *Oncogene* 2002, 21(13):2058-2065.

434. Schiff R, Massarweh SA, Shou J, Bharwani L, Mohsin SK, Osborne CK. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. *Clin Cancer Res* 2004, 10(1 Pt 2):331s-336s.

435. Schlotter CM, Vogt U, Bosse U, Mersch B, Wassmann K. C-myc, not HER-2/neu, can predict recurrence and mortality of patients with node-negative breast cancer. *Breast Cancer Res* 2003, 5(2):R30-36.

436. Schmitt H, Wundrack I, Beck S, Gott P, Welter C, Shizuya H, Simon MI, Blin N. A third P-domain peptide gene (TFF3), human intestinal trefoil factor, maps to 21q22.3. *Cytogenetics and cell genetics* 1996, 72(4):299-302.

437. Schneider BP, Miller KD. Angiogenesis of breast cancer. *J Clin Oncol* 2005, 23(8):1782-1790.

438. Scholz A, Heinze S, Detjen KM, Peters M, Welzel M, Hauff P, Schirner M, Wiedenmann B, Rosewicz S. Activated signal transducer and activator of transcription 3 (STAT3) supports the malignant phenotype of human pancreatic cancer. *Gastroenterology* 2003, 125(3):891-905.

439. Schraufstatter IU, Chung J, Burger M. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. *American journal of physiology Lung cellular and molecular physiology* 2001, 280(6):L1094-1103.

440. Seib T, Blin N, Hilgert K, Seifert M, Theisinger B, Engel M, Dooley S, Zang KD, Welter C. The three human trefoil genes TFF1, TFF2, and TFF3 are located within a region of 55 kb on chromosome 21q22.3. *Genomics* 1997, 40(1):200-202.

441. Seidel HM, Milocco LH, Lamb P, Darnell JE, Jr., Stein RB, Rosen J. Spacing of palindromic half sites as a determinant of selective STAT (signal transducers and activators of transcription) DNA binding and transcriptional activity. *Proc Natl Acad Sci U S A* 1995, 92(7):3041-3045.

442. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997, 33(5):787-791.

443. Shen Y, Devgan G, Darnell JE, Jr., Bromberg JF. Constitutively activated Stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated Stat1. *Proc Natl Acad Sci U S A* 2001, 98(4):1543-1548.

444. Shi Z, Yang WM, Chen LP, Yang DH, Zhou Q, Zhu J, Chen JJ, Huang RC, Chen ZS, Huang RP. Enhanced chemosensitization in multidrug-resistant human breast cancer cells by inhibition of IL-6 and IL-8 production. *Breast Cancer Res Treat* 2012, 135(3):737-747.

445. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Experimental cell research* 2006, 312(5):549-560.

446. Shibuya M. Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis. *BMB Rep* 2008, 41(4):278-286.

447. Shook D, Keller R. Mechanisms, mechanics and function of epithelial–mesenchymal transitions in early development. *Mechanisms of Development* 2003, 120(11):1351-1383.

448. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians* 2014, 64(1):9-29.

449. Silverstein A, Silverstein VB, Nunn LS. Cancer: Conquering a Deadly Disease: Twenty-First Century Books; 2006.

450. Simeone AM, Nieves-Alicea R, McMurtry VC, Colella S, Krahe R, Tari AM. Cyclooxygenase-2 uses the protein kinase C/ interleukin-8/urokinase-type plasminogen activator pathway to increase the invasiveness of breast cancer cells. *Int J Oncol* 2007, 30(4):785-792.

451. Simon AR, Vikis HG, Stewart S, Fanburg BL, Cochran BH, Guan KL. Regulation of STAT3 by direct binding to the Rac1 GTPase. *Science (New York, NY)* 2000, 290(5489):144-147.

452. Singapore Cancer Registry. Interim Annual Registry Report, Trends in Cancer Incidence in Singapore, 2008-2012 2012.

453. Singh RK, Lokeshwar BL. Depletion of intrinsic expression of Interleukin-8 in prostate cancer cells causes cell cycle arrest, spontaneous apoptosis and increases the efficacy of chemotherapeutic drugs. *Mol Cancer* 2009, 8:57.

454. Singh S, Sadanandam A, Nannuru KC, Varney ML, Mayer-Ezell R, Bond R, Singh RK. Small-molecule antagonists for CXCR2 and CXCR1 inhibit human melanoma growth by decreasing tumor cell proliferation, survival, and angiogenesis. *Clin Cancer Res* 2009, 15(7):2380-2386.

455. Singh S, Singh AP, Sharma B, Owen LB, Singh RK. CXCL8 and its cognate receptors in melanoma progression and metastasis. *Future Oncol* 2010, 6(1):111-116.

456. Singletary SE, Allred C, Ashley P, Bassett LW, Berry D, Bland KI, Borgen PI, Clark G, Edge SB, Hayes DF, Hughes LL, Hutter RV, Morrow M, Page DL, Recht A, Theriault RL, Thor A, Weaver DL, Wieand HS, Greene FL. Revision of the American Joint Committee on Cancer staging system for breast cancer. *J Clin Oncol* 2002, 20(17):3628-3636.

457. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science (New York, NY)* 1989, 244(4905):707-712.

458. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001, 344(11):783-792.

459. Sleeman JP, Thiele W. Tumor metastasis and the lymphatic vasculature. *International journal of cancer Journal international du cancer* 2009, 125(12):2747-2756.

460. Smalley M, Ashworth A. Stem cells and breast cancer: A field in transit. *Nat Rev Cancer* 2003, 3(11):832-844.

461. Smid M, Wang Y, Klijn JG, Sieuwerts AM, Zhang Y, Atkins D, Martens JW, Foekens JA. Genes associated with breast cancer metastatic to bone. *J Clin Oncol* 2006, 24(15):2261-2267.

462. Snoussi K, Mahfoudh W, Bouaouina N, Fekih M, Khairi H, Helal AN, Chouchane L. Combined effects of IL-8 and CXCR2 gene polymorphisms on breast cancer susceptibility and aggressiveness. *BMC cancer* 2010, 10:283.

463. Snyder CR, Jiang W, Matsumoto K, Nakamura T, Martin T. Interleukin-8 and Angiogenesis. In: *Growth Factors and their Receptors in Cancer Metastasis.* eds, vol. 2: Springer Netherlands, 2004: pp 51-65.

464. Solomayer EF, Diel IJ, Meyberg GC, Gollan C, Bastert G. Metastatic breast cancer: clinical course, prognosis and therapy related to the first site of metastasis. *Breast Cancer Res Treat* 2000, 59(3):271-278.

465. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, Nordgren H, Farmer P, Praz V, Haibe-Kains B, Desmedt C, Larsimont D, Cardoso F, Peterse H, Nuyten D, Buyse M, Van de Vijver MJ, Bergh J, Piccart M, Delorenzi M. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. *J Natl Cancer Inst* 2006, 98(4):262-272.

466. Sporn MB. The war on cancer. *Lancet* 1996, 347(9012):1377-1381.

467. Stacker SA, Baldwin ME, Achen MG. The role of tumor lymphangiogenesis in metastatic spread. *Faseb j* 2002, 16(9):922-934.

468. Sternlicht MD. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. *Breast Cancer Res* 2006, 8(1):201.

469. Stewart BW, Kleihues P, Organization WH, Cancer IAfRo. World cancer report: Iarc; 2003.

470. Strieter RM, Polverini PJ, Kunkel SL, Arenberg DA, Burdick MD, Kasper J, Dzuiba J, Van Damme J, Walz A, Marriott D, et al. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem* 1995a, 270(45):27348-27357.

471. Strieter RM, Belperio JA, Phillips RJ, Keane MP. CXC chemokines in angiogenesis of cancer. *Semin Cancer Biol* 2004, 14(3):195-200.

472. Strieter RM, Polverini PJ, Arenberg DA, Walz A, Opdenakker G, Van Damme J, Kunkel SL. Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. *Journal of leukocyte biology* 1995b, 57(5):752-762.

473. Strieter RM, Kunkel SL, Elner VM, Martonyi CL, Koch AE, Polverini PJ, Elner SG. Interleukin-8. A corneal factor that induces neovascularization. *The American journal of pathology* 1992, 141(6):1279-1284.

474. Sudhakar A. The matrix reloaded: new insights from type IV collagen derived endogenous angiogenesis inhibitors and their mechanism of action. *J Bioequiv Availab* 2009, 1:52-62.

475. Suemori S, Lynch-Devaney K, Podolsky DK. Identification and characterization of rat intestinal trefoil factor: tissue- and cell-specific member of the trefoil protein family. *Proc Natl Acad Sci U S A* 1991, 88(24):11017-11021.

476. Sun Y, Wang L, Zhou Y, Mao X, Deng X. Cloning and characterization of the human trefoil factor 3 gene promoter. *PLoS One* 2014, 9(4):e95562.

477. Sundar SS, Ganesan TS. Role of Lymphangiogenesis in Cancer. *Journal of Clinical Oncology* 2007, 25(27):4298-4307.

478. Tabruyn SP, Griffioen AW. Molecular pathways of angiogenesis inhibition. *Biochem Biophys Res Commun* 2007, 355(1):1-5.

479. Talmadge JE, Fidler IJ. AACR Centennial Series: The Biology of Cancer Metastasis: Historical Perspective. *Cancer research* 2010, 70(14):5649-5669.

480. Tan XD, Hsueh W, Chang H, Wei KR, Gonzalez-Crussi F. Characterization of a putative receptor for intestinal trefoil factor in rat small intestine: identification by in situ binding and ligand blotting. *Biochem Biophys Res Commun* 1997, 237(3):673-677.

481. Tan XD, Liu QP, Hsueh W, Chen YH, Chang H, Gonzalez-Crussi F. Intestinal trefoil factor binds to intestinal epithelial cells and induces nitric oxide production: priming and enhancing effects of mucin. *The Biochemical journal* 1999, 338 (Pt 3):745-751.

482. Taupin D, Wu DC, Jeon WK, Devaney K, Wang TC, Podolsky DK. The trefoil gene family are coordinately expressed immediate-early genes: EGF receptor- and MAP kinase-dependent interregulation. *J Clin Invest* 1999, 103(9):R31-38.

483. Taupin D, Podolsky DK. Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* 2003, 4(9):721-732.

484. Taupin DR, Kinoshita K, Podolsky DK. Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proc Natl Acad Sci U S A* 2000a, 97(2):799-804.

485. Taupin DR, Kinoshita K, Podolsky DK. Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* 2000b, 97(2):799-804.

486. Tebbutt NC, Giraud AS, Inglese M, Jenkins B, Waring P, Clay FJ, Malki S, Alderman BM, Grail D, Hollande F, Heath JK, Ernst M. Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. *Nat Med* 2002, 8(10):1089-1097.

487. Teo MCC, Soo KC. Cancer Trends and Incidences in Singapore. *Japanese Journal of Clinical Oncology* 2013, 43(3):219-224.

488. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009, 139(5):871-890.

489. Thim L, May FE. Structure of mammalian trefoil factors and functional insights. *Cell Mol Life Sci* 2005, 62(24):2956-2973.

490. Thim L. A new family of growth factor-like peptides. 'Trefoil' disulphide loop structures as a common feature in breast cancer associated peptide (pS2), pancreatic spasmolytic polypeptide (PSP), and frog skin peptides (spasmolysins). *FEBS Lett* 1989, 250(1):85-90.

491. Thim L, Thomsen J, Christensen M, Jorgensen KH. The amino acid sequence of pancreatic spasmolytic polypeptide. *Biochim Biophys Acta* 1985, 827(3):410-418.

492. Thull DL, Vogel VG. Recognition and management of hereditary breast cancer syndromes. *Oncologist* 2004, 9(1):13-24.

493. Tiede B, Kang Y. From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. *Cell Res* 2011, 21(2):245-257.

494. Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991, 266(18):11947-11954.

495. Todorović-Raković N, Milovanović J. Interleukin-8 in breast cancer progression. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 2013, 33(10):563-570.

496. Tomasetto C, Rio MC, Gautier C, Wolf C, Hareuveni M, Chambon P, Lathe R. hSP, the domain-duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma. *Embo j* 1990, 9(2):407-414.

497. Trevino JG, Gray MJ, Nawrocki ST, Summy JM, Lesslie DP, Evans DB, Sawyer TK, Shakespeare WC, Watowich SS, Chiao PJ, McConkey DJ, Gallick GE. Src activation of Stat3 is an independent requirement from NF-kappaB activation for constitutive IL-8 expression in human pancreatic adenocarcinoma cells. *Angiogenesis* 2006, 9(2):101-110.

498. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998, 93(5):705-716.

499. Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* 1998, 18(5):2545-2552.

500. Uchino H, Kataoka H, Itoh H, Hamasuna R, Koono M. Overexpression of intestinal trefoil factor in human colon carcinoma cells reduces cellular growth in vitro and in vivo. *Gastroenterology* 2000, 118(1):60-69.

501. Vadeboncoeur N, Segura M, Al-Numani D, Vanier G, Gottschalk M. Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by Streptococcus suis serotype 2. *FEMS immunology and medical microbiology* 2003, 35(1):49-58.

502. Vajkoczy P, Menger MD, Vollmar B, Schilling L, Schmiedek P, Hirth KP, Ullrich A, Fong TAT. Inhibition of Tumor Growth, Angiogenesis, and Microcirculation by the Novel Flk-1 Inhibitor SU5416 as Assessed by Intravital Multi-fluorescence Videomicroscopy. *Neoplasia* 1999, 1(1):31-41.

503. Valastyan S, Weinberg Robert A. Tumor Metastasis: Molecular Insights and Evolving Paradigms. *Cell*, 147(2):275-292.

504. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell* 2011, 147(2):275-292.

505. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, Sharma N, Dekoninck S, Blanpain C. Distinct stem cells contribute to mammary gland development and maintenance. *Nature* 2011, 479(7372):189-193.

506. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. *Annual review of pharmacology and toxicology* 1998, 38:97-120.

507. Vargo-Gogola T, Rosen JM. Modelling breast cancer: one size does not fit all. *Nat Rev Cancer* 2007, 7(9):659-672.

508. Varmus H, Weinberg RA. Genes and the Biology of Cancer: Scientific American Library; 1993.

509. Varney ML, Johansson SL, Singh RK. Distinct expression of CXCL8 and its receptors CXCR1 and CXCR2 and their association with vessel density and aggressiveness in malignant melanoma. *Am J Clin Pathol* 2006, 125(2):209-216.

510. Vennstrom B, Sheiness D, Zabielski J, Bishop JM. Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. *Journal of virology* 1982, 42(3):773-779.

511. Visvader JE, Lindeman GJ. The unmasking of novel unipotent stem cells in the mammary gland. *The EMBO Journal* 2011, 30(24):4858-4859.

512. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 2002, 20(3):719-726.

513. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004, 10(8):789-799.

514. Waugh DJJ, Wilson C. The Interleukin-8 Pathway in Cancer. *Clinical Cancer Research* 2008, 14(21):6735-6741.

515. Wei D, Le X, Zheng L, Wang L, Frey JA, Gao AC, Peng Z, Huang S, Xiong HQ, Abbruzzese JL, Xie K. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. *Oncogene* 2003a, 22(3):319-329.

516. Wei LH, Kuo ML, Chen CA, Chou CH, Lai KB, Lee CN, Hsieh CY. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene* 2003b, 22(10):1517-1527.

517. Weigelt B, Verduijn P, Bosma AJ, Rutgers EJ, Peterse HL, van't Veer LJ. Detection of metastases in sentinel lymph nodes of breast cancer patients by multiple mRNA markers. *Br J Cancer* 2004, 90(8):1531-1537.

518. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* 2011, 17(11):1359-1370.

519. Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ. Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* 2000, 20(16):5797-5807.

520. Westley BR, Griffin SM, May FE. Interaction between TFF1, a gastric tumor suppressor trefoil protein, and TFIZ1, a brichos domain-containing protein with homology to SP-C. *Biochemistry* 2005, 44(22):7967-7975.

521. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 2008, 27(45):5904-5912.

522. Williams KL, Fuller CR, Dieleman LA, DaCosta CM, Haldeman KM, Sartor RB, Lund PK. Enhanced survival and mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth hormone. *Gastroenterology* 2001, 120(4):925-937.

523. Wilson KS, Roberts H, Leek R, Harris AL, Geradts J. Differential gene expression patterns in HER2/neu-positive and -negative breast cancer cell lines and tissues. *The American journal of pathology* 2002, 161(4):1171-1185.

524. Woodward TL, Xie JW, Haslam SZ. The role of mammary stroma in modulating the proliferative response to ovarian hormones in the normal mammary gland. *Journal of mammary gland biology and neoplasia* 1998, 3(2):117-131.

525. Wright NA, Hoffmann W, Otto WR, Rio MC, Thim L. Rolling in the clover: trefoil factor family (TFF)-domain peptides, cell migration and cancer. *FEBS Lett* 1997, 408(2):121-123.

526. Wright NA. Aspects of the biology of regeneration and repair in the human gastrointestinal tract. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 1998, 353(1370):925-933.

527. Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev* 2001, 12(4):375-391.

528. Xu L, Fidler IJ. Interleukin 8: an autocrine growth factor for human ovarian cancer. *Oncol Res* 2000, 12(2):97-106.

529. Xu XQ, Emerald BS, Goh EL, Kannan N, Miller LD, Gluckman PD, Liu ET, Lobie PE. Gene expression profiling to identify oncogenic determinants of autocrine human growth hormone in human mammary carcinoma. *J Biol Chem* 2005, 280(25):23987-24003.

530. Yamachika T, Werther JL, Bodian C, Babyatsky M, Tatematsu M, Yamamura Y, Chen A, Itzkowitz S. Intestinal trefoil factor: a marker of poor prognosis in gastric carcinoma. *Clin Cancer Res* 2002, 8(5):1092-1099.

531. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004, 117(7):927-939.

532. Yang XD, Corvalan JR, Wang P, Roy CM, Davis CG. Fully human anti-interleukin-8 monoclonal antibodies: potential therapeutics for the treatment of inflammatory disease states. *Journal of leukocyte biology* 1999, 66(3):401-410.

533. Yang XN, Lu YP, Liu JJ, Huang JK, Liu YP, Xiao CX, Jazag A, Ren JL, Guleng B. Piezo1 Is as a Novel Trefoil Factor Family 1 Binding Protein that Promotes Gastric Cancer Cell Mobility In Vitro. *Digestive diseases and sciences* 2014.

534. Yip KW, Reed JC. Bcl-2 family proteins and cancer. *Oncogene* 2008, 27(50):6398-6406.

535. Yong Z, Lin W, Yong S, Guang-Ping L, Dan W, Shang-Jun L, Wei W, Xi P. Kinetic characterization of an intestinal trefoil factor receptor. *PLoS One* 2013, 8(9):e74669.

536. Young MR, Young ME, Lozano Y, Bagash JM. Activation of protein kinase A increases the in vitro invasion, migration, and adherence to reconstituted basement membrane by Lewis lung carcinoma tumor cells. *Invasion & metastasis* 1992, 12(5-6):253-263.

537. Youngs SJ, Ali SA, Taub DD, Rees RC. Chemokines induce migrational responses in human breast carcinoma cell lines. *International journal of cancer Journal international du cancer* 1997, 71(2):257-266.

538. Yuan A, Chen JJ, Yao PL, Yang PC. The role of interleukin-8 in cancer cells and microenvironment interaction. *Frontiers in bioscience : a journal and virtual library* 2005, 10:853-865.

539. Zhou BP, Hung MC. Dysregulation of cellular signaling by HER2/neu in breast cancer. *Seminars in oncology* 2003, 30(5 Suppl 16):38-48.

540. Zhou S, Wang GP, Liu C, Zhou M. Eukaryotic initiation factor 4E (eIF4E) and angiogenesis: prognostic markers for breast cancer. *BMC cancer* 2006, 6:231.

541. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000, 12(2):121-127.

Appendices

Preparation of buffers

Experiment	Buffers and solutions	Composition
Bacterial Transformation	LB medium	10g Bacto™Tryptone, 5g Bacto™Yeast extract, 5g NaCl, 1L Milli-Q H_2O, autoclaved and stored at room temperature
	LB plate	15g agarose was added to 1L liquid LB medium and sterilized by autoclaving. After the medium was cooled down to 50ºC, ampicillin was added at the concentration of 100 μg/mL. After gently mixing, the medium was poured into the sterile plates. Upon the medium was solidified, the dishes were stored at 4ºC in the inverted position.
Cell culture	RPMI Medium	10.39g/L RPMI powder, 2g/L $NaHCO_4$; Dissolve it by adding autoclaved H_20, adjust the pH to 7.2 by adding HCl or NaOH, adjust volume to 1L with additional autoclaved H_20 and filter sterilize.
	Advanced DMEM-F12	Advanced DMEM-F12 media supplemented with 10 mM L-Glutamine, penicillin/streptomycin and 10% Fetal Bovine Serum
	Complete RPMI medium	450 mL RPMI medium, 50ml heat-inactivated fetal bovine serum, 5mL glutamine, 5mL penicillin/streptomycin
	10x PBS	80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4, 1L Milli-Q H_2O. Adjust pH to 7.4
	10x trypsin/EDTA solution (0.25%)	2.5g Trypsin, 0.372g EDTA, 0.35g $NaHCO_3$, 1L autoclaved H_2O. Adjust pH to 7.2.
	HEPES buffer	0.8766 g NaCl, 0.47688 g HEPES, 100 mL MilliQ H_2O. Adjust pH value to 7.4 and sterilize by 0.2 μm filter
	HBSS	0.4g/L Potassium Chloride; 0.06 g/L Potassium Phosphate Monobasic (anhydrous); 8 g/L Sodium Chloride; 0.04788 g/L Sodium Phosphate Dibasic (anhydrous); 1g/L D-Glucose
	Freezing medium	1 mL DMSO, 20 mL heat inactivated fetal bovine serum
	0.4% trypan blue	0.4g Trypan blue, 100 mL 1x PBS.
RNA extraction	DEPC water	1 mL DEPC, 1L Milli-Q H_2O. Solution was

		made in the fume hood and stirred overnight at room temperature before being autoclaved.
DNA electrophoresis	50x TAE buffer	242g Tris, 37.2g $Na_2EDTA.2H_2O$, 57.2 mL glacial acetic acid, 1L Milli-Q H_2O
	10x TBE buffer	108g Tris, 55g boric acid, 7.44g EDTA (pH 8.0), 1 L H_2O
	1-2% agarose gel	1-2g agarose, 100 mL l 1x TAE buffer or TBE buffer
	1% Ethidium bromide solution	0.2g ethidium bromide, 20 mL Milli-Q H_2O
	DNA loading dye 6x	250 mg bromophenol, 30ml glycerol, 70 mL H_2O
Protein extraction	Lysis buffer	10 mL (final concentration): 500 µL (1 M Tris-HCl pH 7.4 (121.1 g/mol)); 1 mL (10% Nonidet P-40); 300 µL (5 M NaCl (58.44 g/mol)); 50 µL (0.2 M EDTA (452.24 g/mol)); 1mM NaF; 1mM PMSF; 100 µL (100 mM Na_3VO_4); 1.43 mL(7x protease inhibitor stock); 6.62 mL H_20
SDS-PAGE	4% Stacking gel	500µl 40% acrylamide, 1.26 mL 0.5M Tris-HCl pH 6.8, 50 µL 10% SDS, 3.18 mL Milli-Q H_20, 5 µL TEMED, 25 µL 10% APS
	12% Separating gel	3 mL 40% acrylamide, 2.5 mL 1.5 M Tris-HCl pH 8.8, 100 µL 10% SDS, 4.35 mL Milli-Q H_20, 5 µL TEMED, 50 µL 10% APS
	6x SDS loading dye	6 mL glycerol, 3 mL 1M Tris-HCl, pH 6.8, 1.2g SDS and 5mg bromophenol blue
	1x SDS running buffer	3.03g Tris, 14.41g glycine, 10ml 10% SDS, 1L Milli-Q H_20
	1x Transfer buffer	3.03g Tris, 14.41g glycine, 200 mL MeOH, 800 mL Milli-Q H_20
Western blot	0.1% Tween-20 PBS	100 mL 10x PBS, 900 mL MilliQ H_20, 1 mL Tween-20
	Blocking buffer	5g non-fat dry milk powder, 100 mL 0.1% Tween-20 PBS
	Commassie Blue Stain	2g Commassie Blue; 500 mL MeOH; 70 mL Acetic acid, in 1L H_20
	Commassie destain	95 mL MeOH; 75mL Glacial acetic acid; Made up to 1L with MilliQ H_20
Precast gel Western blot	1x running buffer	1x MOPS SDS buffer (20x) in 600 mL MilliQ H_20
	1X transfer buffer	1x transfer buffer (20x); 10%/20% MeOH; in 500ml MilliQ H_20
BrdU assay	Blocking solution	2% Horse serum in 1x PBS

	Primary Antibody Solution	Anti-BrdU antibody (1:100) in 10 mL PBS with 2% horse serum
	Secondary Antibody Solution	Secondary Antibody (1:200) in 10 mL PBS with 0.2% Triton (2µL) and 2% horse serum
	Tertiary Antibody solution	1 drop Reagent A; 1 drop reagent B; 10 mL 1x PBS
	Substrate solution	1 DAB tablet; 1 H_20_2 tablet; 1 mL MilliQ H_20
Others	1.5 M Tris-HCl	27.3g Tris; 80 mL MilliQ H_20 ; Adjust pH value to 8.8 with 2N HCl and make up to 150 mL with MilliQ H_20
	0.5M Tris HCL	9.1 g Tris; 80 mL MilliQ H_20 ; Adjust pH value to 6.8 with 2N HCl and make up to 150 mL with MilliQ H_20
	TE buffer	10mM Tris-HCl ; 1mM EDTA pH 8.0
	4x Electrophoresis stock buffer	57.4g Glycine; 7g Tris; 4g SDS; make up to 1L with MilliQ H_20
	Anesthetic for *in vivo*	10 mL: 1 mL of 100 mg/mL ketamine, 0.5 mL of 2% xylazine and 8.5 mL MilliQ H_20

Copyright

Figure 1

ELSEVIER LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Lau Wai Hoe
Customer address	National University of Singapore, Singapore, None 117599
License number	3438291264364
License date	Jul 29, 2014
Licensed content publisher	Elsevier
Licensed content publication	Cell
Licensed content title	The Hallmarks of Cancer
Licensed content author	Douglas Hanahan,Robert A Weinberg
Licensed content date	7 January 2000
Licensed content volume number	100
Licensed content issue number	1
Number of pages	14
Start Page	57
End Page	70
Type of Use	Thesis/Dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Elsevier VAT number	GB 494 6272 12

Figure 2

ELSEVIER LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.
All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Lau Wai Hoe
Customer address	National University of Singapore, Singapore, 117599
License number	3438300728919
License date	Jul 29, 2014
Licensed content publisher	Elsevier
Licensed content publication	Cell
Licensed content title	Hallmarks of Cancer: The Next Generation
Licensed content author	Douglas Hanahan,Robert A. Weinberg
Licensed content date	4 March 2011
Licensed content volume number	144
Licensed content issue number	5
Number of pages	29
Start Page	646
End Page	674
Type of Use	Thesis/Dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Elsevier VAT number	GB 494 6272 12

Figure 3

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438310350637
License date	Jul 29, 2014
Order Content Publisher	Nature Publishing Group
Order Content Publication	Nature Reviews Cancer
Order Content Title	The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited
Order Content Author	Isaiah J. Fidler
Order Content Date	Jun 1, 2003
Volume number	3
Issue number	6
Type of Use	Thesis/Dissertation
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	2
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)
   Note: For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)
6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:
7. Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
8. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:
   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).
   Note: For translation from the *British Journal of Cancer*, the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Figure 4

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438541451598
License date	Jul 29, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	The EMBO Journal
Licensed content title	The unmasking of novel unipotent stem cells in the mammary gland
Licensed copyright line	Copyright © 2011 European Molecular Biology Organization
Licensed content author	Jane E Visvader,Geoffrey J Lindeman
Licensed content date	Nov 8, 2011
Start page	4858
End page	4859
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	1
Will you be translating?	No
Title of your thesis / dissertation	The role of Trefoil Factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Expected size (number of pages)	300
Total	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS
This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking accept in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone

basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.
- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.
- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU
- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY

NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCCs Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCCs Billing and Payment terms and conditions, these terms and conditions shall prevail.
- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCCs Billing and Payment terms and conditions.
- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.
- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such states conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

Figure 8

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438301223900
License date	Jul 29, 2014
Order Content Publisher	Nature Publishing Group
Order Content Publication	Nature
Order Content Title	The microenvironment of the tumour-host interface
Order Content Author	Lance A. Liotta and Elise C. Kohn
Order Content Date	May 17, 2001
Volume number	411
Issue number	6835
Type of Use	Thesis/Dissertation
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Figures	1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Total	0.00 USD
Terms and Conditions	

Figure 10

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3442550799846
License date	Aug 05, 2014
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Medicine
Licensed content title	Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression
Licensed content author	Mina J Bissell, William C Hines
Licensed content date	Mar 7, 2011
Volume number	17
Issue number	3
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is s equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be

obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)
   Note: For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)
6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:
   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:
   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).
   Note: For translation from the *British Journal of Cancer*, the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Figure 11

SPRINGER LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438871351683
License date	Jul 30, 2014
Order Content Publisher	Springer
Order Content Publication	Cellular and Molecular Life Sciences
Order Content Title	Trefoil factors
Order Content Author	L. Thim
Order Content Date	Jan 1, 2005
Volume number	62
Issue number	24
Type of Use	Thesis/Dissertation
Portion	Figures
Author of this Springer article	No
Order reference number	None
Original figure numbers	1
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size(pages)	300
Total	0.00 USD

Terms and Conditions

Introduction

The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License

With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the university's intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis limited to university-use only. If the thesis is going to be published, permission needs to be re-obtained (selecting "book/textbook" as the type of use).

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, subject to a courtesy information to the author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for

Figure 12, 13

ELSEVIER LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Lau Wai Hoe
Customer address	National University of Singapore, Singapore, None 117599
License number	3438880191889
License date	Jul 30, 2014
Licensed content publisher	Elsevier
Licensed content publication	Biochimica et Biophysica Acta (BBA) - Reviews on Cancer
Licensed content title	The pS2/TFF1 trefoil factor, from basic research to clinical applications
Licensed content author	Stéphane Ribieras,Catherine Tomasetto,Marie-Christine Rio
Licensed content date	19 August 1998
Licensed content volume number	1378
Licensed content issue number	1
Number of pages	17
Start Page	F61
End Page	F77
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	3
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Elsevier VAT number	GB 494 6272 12
Price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account

and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure

to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

For journal authors: the following clauses are applicable in addition to the above: Permission granted is limited to the author accepted manuscript version* of your paper.

***Accepted Author Manuscript (AAM) Definition:** An accepted author manuscript (AAM) is the author's version of the manuscript of an article that has been accepted for publication and which may include any author-incorporated changes suggested through the processes of submission processing, peer review, and editor-author communications. AAMs do not include other publisher value-added contributions such as copy-editing, formatting, technical enhancements and (if relevant) pagination.

You are not allowed to download and post the published journal article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Posting to a repository: Authors may post their AAM immediately to their employer's institutional repository for internal use only and may make their manuscript publically available after the journal-specific embargo period has ended.

Please also refer to Elsevier's Article Posting Policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only.. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the

Figure 14

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438880666212
License date	Jul 30, 2014
Order Content Publisher	Nature Publishing Group
Order Content Publication	Nature Reviews Molecular Cell Biology
Order Content Title	Trefoil factors: initiators of mucosal healing
Order Content Author	Douglas Taupin and Daniel K. Podolsky
Order Content Date	Sep 1, 2003
Volume number	4
Issue number	9
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Total	0.00 USD

Terms and Conditions

Figure 17

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438890294613
License date	Jul 30, 2014
Order Content Publisher	Nature Publishing Group
Order Content Publication	Nature Reviews Cancer
Order Content Title	Tumorigenesis and the angiogenic switch
Order Content Author	Gabriele Bergers and Laura E. Benjamin
Order Content Date	Jun 1, 2003
Volume number	3
Issue number	6
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	1
Author of this NPG article	no
Your reference number	None
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Total	0.00 USD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)
   Note: For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)
6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:
   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.
   Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:
   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).
   Note: For translation from the *British Journal of Cancer*, the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Figure 20

AMERICAN ASSOCIATION FOR CANCER RESEARCH LICENSE TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and American Association for Cancer Research ("American Association for Cancer Research") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Association for Cancer Research, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3438890965391
License date	Jul 30, 2014
Order Content Publisher	American Association for Cancer Research
Order Content Publication	Clinical Cancer Research
Order Content Title	The Interleukin-8 Pathway in Cancer
Order Content Author	David J.J. Waugh, Catherine Wilson
Order Content Date	November 1, 2008
Volume number	14
Issue number	21
Type of Use	Thesis/Dissertation
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation	1
Territory of distribution	Worldwide
Title of your thesis / dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Total	0.00 USD
Terms and Conditions	

American Association for Cancer Research (AACR) Terms and Conditions

INTRODUCTION
The Publisher for this copyright material is the American Association for Cancer Research (AACR). By clicking "accept" in connection with completing this licensing transaction, you agree to the following terms and conditions applying to this transaction. You also agree to the Billing and Payment terms and conditions established by Copyright Clearance Center (CCC) at the time you opened your Rightslink account.

LIMITED LICENSE
The AACR grants exclusively to you, the User, for onetime, non-exclusive use of this material for the purpose stated in your request and used only with a maximum distribution equal to the number you identified in the permission process. Any form of republication must be completed within one year although copies made before then may be distributed thereafter and any electronic posting is limited to a period of one year. Reproduction of this material is confined to the purpose and/or media for which permission is granted. Altering or modifying this material is not permitted. However, figures and illustrations may be minimally altered or modified to serve the new work.

GEOGRAPHIC SCOPE
Licenses may be exercised as noted in the permission process

RESERVATION OF RIGHTS
The AACR reserves all rights not specifically granted in the combination of 1) the license details provided by you and accepted in the course of this licensing transaction, 2) these terms and conditions , and 3) CCC's Billing and Payment terms and conditions.
DISCLAIMER
You may obtain permission via Rightslink to use material owned by AACR. When you are requesting permission to reuse a portion for an AACR publication, it is your responsibility to examine each portion of content as published to determine whether a credit to, or copyright notice of a third party owner is published next to the item. You must obtain permission from the third party to use any material which has been reprinted with permission from the said third party. If you have not obtained permission from the third party, AACR disclaims any responsibility for the use you make of items owned by them.
LICENSE CONTINGENT ON PAYMENT
While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you, either by the publisher or by the CCC, as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions, or any of the CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and the publisher reserves the right to take any and all action to protect its copyright in the materials.
COPYRIGHT NOTICE
You must include the following credit line in connection with your reproduction of the licensed material: "Reprinted (or adapted) from Publication Title, Copyright Year, Volume/Issue, Page Range, Author, Title of Article, with permission from AACR".
TRANSLATION
This permission is granted for non-exclusive world English rights only.
WARRANTIES
Publisher makes no representations or warranties with respect to the licensed material.
INDEMNIFICATION
You hereby indemnify and agree to hold harmless the publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
REVOCATION
The AACR reserves the right to revoke a license for any reason, including but not limited to advertising and promotional uses of AACR content, third party usage and incorrect figure source attribution.
NO TRANSFER OF LICENSE
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
NO AMENDMENT EXCEPT IN WRITING
This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
OBJECTION TO CONTRARY TERMS
Publishers hereby objects to any terms contained in any purchase order, acknowledgement, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions, and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
THESIS/DISSERTATION TERMS
If your request is to reuse an article authored by you and published by the AACR in your dissertation/thesis, your thesis may be submitted to your institution in either in print or electronic

form. Should your thesis be published commercially, please reapply.

ELECTRONIC RESERVE

If this license is made in connection with a course, and the Licensed Material or any portion thereof is to be posted to a website, the website is to be password protected and made available only to the students registered for the relevant course. The permission is granted for the duration of the course. All content posted to the website must maintain the copyright information notice.

JURISDICTION

This license transaction shall be governed by and construed in accordance with the laws of Pennsylvania. You hereby agree to submit to the jurisdiction of the federal and state courts located in Pennsylvania for purposes of resolving any disputes that may arise in connection with this licensing transaction.

Figure 21

ELSEVIER LICENSE
TERMS AND CONDITIONS

This is a License Agreement between Lau Wai Hoe ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Lau Wai Hoe
Customer address	National University of Singapore, Singapore, None 117599
License number	3438891063792
License date	Jul 30, 2014
Licensed content publisher	Elsevier
Licensed content publication	Cancer Cell
Licensed content title	Antiangiogenic Therapy Elicits Malignant Progression of Tumors to Increased Local Invasion and Distant Metastasis
Licensed content author	Marta Pàez-Ribes,Elizabeth Allen,James Hudock,Takaaki Takeda,Hiroaki Okuyama,Francesc Viñals,Masahiro Inoue,Gabriele Bergers,Douglas Hanahan,Oriol Casanovas
Licensed content date	3 March 2009
Licensed content volume number	15
Licensed content issue number	3
Number of pages	12
Start Page	220
End Page	231
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Title of your thesis/dissertation	The role of Trefoil factor 3 (TFF3) in mammary carcinoma angiogenesis
Expected completion date	Aug 2014
Estimated size (number of pages)	300
Elsevier VAT number	GB 494 6272 12
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection

with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in

this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

For journal authors: the following clauses are applicable in addition to the above: Permission granted is limited to the author accepted manuscript version* of your paper.

***Accepted Author Manuscript (AAM) Definition:** An accepted author manuscript (AAM) is the author's version of the manuscript of an article that has been accepted for publication and which may include any author-incorporated changes suggested through the processes of submission processing, peer review, and editor-author communications. AAMs do not include other publisher value-added contributions such as copy-editing, formatting, technical enhancements and (if relevant) pagination.

You are not allowed to download and post the published journal article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Posting to a repository: Authors may post their AAM immediately to their employer's institutional repository for internal use only and may make their manuscript publically available after the journal-specific embargo period has ended.

Please also refer to Elsevier's Article Posting Policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only.. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published

commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.